CIRCULATION IN *HALIOTIS IRIS* – APPLICATION OF FLUORESCENT MICROSPHERE TECHNIQUES

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Haliotis iris

TABLE OF CONTENTS

CHAPTER THREE: CIRCULATORY CHANGES DURING EMERSION

CHAPTER FOUR: CIRCULATORY CHANGES DURING CLAMPING

LIST OF FIGURES

CHAPTER ONE:

CHAPTER TWO:

CHAPTER THREE:

CHAPTER FOUR:

LIST OF TABLES

CHAPTER TWO:

CHAPTER FOUR:

ABSTRACT

The present study used fluorescent microspheres to examine the circulation in *Haliotis iris*. Fluorescent microspheres have not been used previously to study the circulatory system of invertebrates, despite their extensive application in the field of vertebrate circulatory physiology.

A protocol was developed for the injection of fluorescent microspheres into *H. iris* to examine their distribution to organs by the circulatory system. Injected microspheres mixed into the blood and were distributed to organs of the animal in proportion to the relative blood flow each received.

A method of recovery and quantification of injected microspheres from the organs of *H. iris* was employed and refined. Satisfactory recoveries of the injected labels using the modified recovery method were achieved (>90%), and the relative distribution of the blood flow to the organs in resting animals was able to be determined.

Circulatory changes associated with the physiological states of clamping and long-term (24hrs) emersion were investigated by sequentially injecting differently labelled microspheres under control and treatment states. The relative entrapment of the injected labels in the organs under the different physiological states reflected circulatory changes which these states produce.

During emersion, increases in the relative perfusion of peripheral tissues which facilitate auxiliary gas exchange in other gastropods were not observed in *H. iris*. A decrease in relative perfusion of the foot suggested that this muscle may be isolated from the main circulation during emersion, and the simultaneous increase in microsphere entrapment observed in the gills but not other predominantly venous tissues, suggested arterial blood is, at least in part, bypassing the conventionally defined routes of flow during this physiological state.

Clamping was associated with a decrease in relative perfusion of the foot muscle and more noticeably the adductor muscle, both of which appeared to be isolated from the rest of the

circulation, and a simultaneous increase in microsphere entrapment in the gills strongly supports the existence of a hypothesised arterio-venous shunt.

A protocol for measurement of cardiac output and stroke volume in *H. iris* using a modified reference sampling method was developed. Cardiac output and stroke volume (CSV) was estimated in resting animals and changes in the cardiac output associated with clamping were investigated.

Cardiac output was estimated at around 120ml·kg^{-1}·min^{-1}, and CSV was around 1.2ml in the resting animals examined.

A considerable decrease in cardiac output to around 40 ml·kg^{-1}·min^{-1} was observed when the animals were forced to clamp. This decrease was due to reductions in both heart rate and cardiac stroke volume.

The method for using fluorescent microspheres to measure regional perfusion in *H. iris* was validated by comparison of control and treatment data and histological methods.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 *HALIOTIS IRIS* (PAUA)

The black footed abalone *Haliotis iris* Martyn, usually referred to by their Maori name "paua" are the largest and most common of the 3 species of abalone in New Zealand (Schiel, 1992). *H. iris* are macroalgivorous, feeding on both suspended and attached seaweed (Allen et al., 2001; Poore, 1972a). They are found on rocky reefs and ledges in the lower intertidal and subtidal zones (Poore, 1972b) usually at depths of less than 5m (Schiel and Breen, 1991).

H. iris is of considerable importance to New Zealand with annual commercial landings of 1200 metric tonnes (Andrew et al., 2000), making it one of the largest abalone fisheries in the world (Schiel and Breen, 1991). The value of the fishery extends beyond commercial significance to both recreational and indigenous fishing, both in culinary and aesthetic aspects.

In 1986 the implementation of the fisheries quota management system (QMS) in New Zealand saw the paua fishery more carefully regulated with a total allowable commercial catch (TACC) set in each of the seven Quota Managed Areas (QMAs) (Schiel and Breen, 1991).

A considerable amount of research on *H. iris* has focussed on ecological aspects of this species, such as distribution, growth, reproduction and mortality rates, with a view to monitoring TACCs, by establishing the biomass required for maximum sustainable yield (B_{msy}), thus ascertaining the health and long-term viability of the fishery (Andrew et al., 2000; Kendrick and Andrew, 2000; Ministry of Fisheries, 2002; Poore, 1972a; 1972b; 1972c; 1973; Sainsbury, 1982a; 1982b; Schiel and Breen, 1991).

Worldwide there has been a 30 percent decrease in abalone fishing from 1989 to 1999 (Gordon and Cook, 2001), with many fisheries severely depleted. The Californian abalone industry (*H. rufescens*) is closed until at least 2008 and the South African abalone (*H. midae*)

season has been shortened considerably after continued heavy poaching. In New Zealand a steady decline in catch per unit effort (CPUE) has been observed in all the QMAs since 1983 (Kendrick and Andrew, 2000).

The worldwide abalone supply from fishery is not expected to increase and the worldwide demand in 2004 is expected to stay about the same as at present, in excess of 20,000 metric tonnes (Kendrick and Andrew, 2000). The current (1999) total supply of abalone worldwide is around 7000 metric tonnes short of the demand, and in 2004 is estimated at 15,000 metric tonnes, a shortfall of 5,000 metric tonnes (Kendrick and Andrew, 2000).

In contrast to the decrease in worldwide abalone fishing, production by aquacultural methods has increased 600 percent from 1989 to 1999 (Kendrick and Andrew, 2000). It is hardly surprising that in more recent years the focus of worldwide research on *Haliotis sp.* has been on aquaculture aspects, such as maximising settlement, growth rates and improving farming techniques (Allen et al., 2001; Jackson et al., 2001; Servier-Zaragoza et al., 2001; Shipton and Britz, 2001). There is also a steady interest in live abalone, which are shipped mostly to Asian countries which greatly dominate the demand market (Gordon and Cook, 2001).

Due to the intense increase in the aquaculture of *H. iris* in New Zealand and its geographical distance from Asia when considering live-freighting of specimens, a good understanding of the physiology of this animal and its response to commercial processes would be beneficial. The physiological consequences of hypoxia due to emersion, or clamping, a reaction of the animal whenever it is startled, are of interest in any venture where handling or transport of *H. iris* is required. Knowledge of the design and functioning of the circulatory system is central to understanding the effects of a wide range of external stresses on physiological processes (excretion, gas exchange, nutrition etc).

1.2 ABALONE CIRCULATION

The circulatory system in abalone is characterised as an open system, having an incomplete system of blood vessels (Bourne et al., 1990; Russell and Evans, 1989; Withers, 1992) and tissues are bathed with blood as it percolates through a system of sinuses and lacunar tissue spaces (LTS).

Our understanding of the physiological aspects of the circulatory system in haliotids is limited. The reader is directed to a thorough study by Crofts (1929) on *H. tuberculata* for detailed descriptions of the circulatory anatomy.

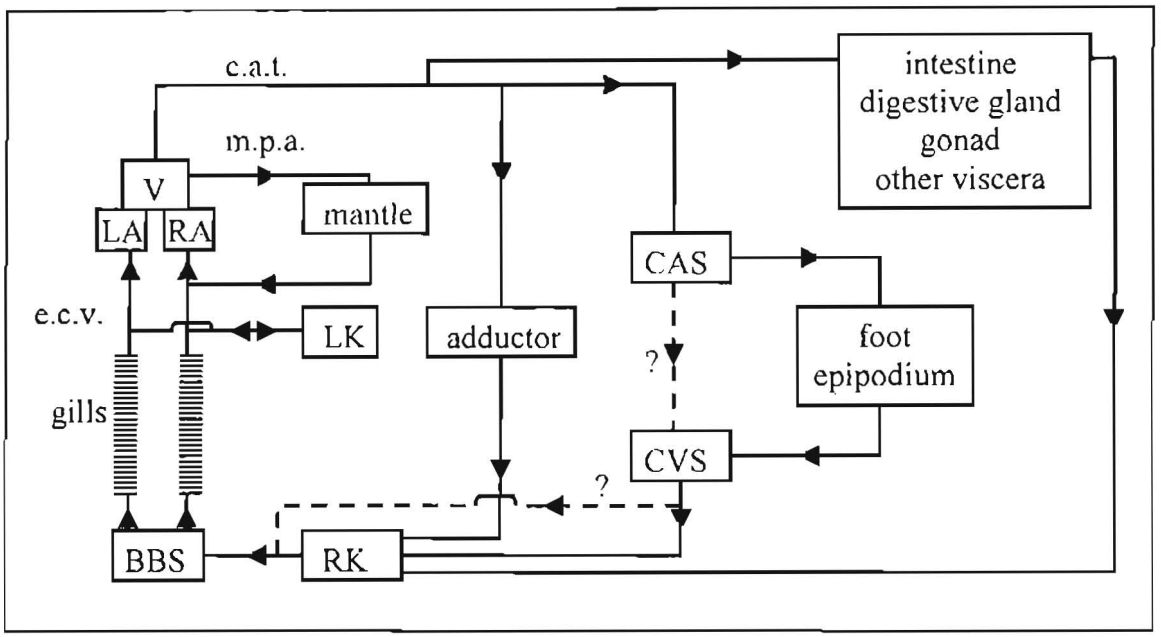


Figure 1.1. Outline of the circulatory system in *Haliotis sp.* The blood flow is indicated by the direction of the arrows, known routes of blood blow by solid lines and hypothesised or uncertain routes by dashed lines. The organs listed are: left atrium (LA), right atrium (RA), ventricle (V), cephalo-arterial sinus (CAS), cephalo-venous sinus (CVS), right kidney (RK), basibranchial sinus (BBS), left kidney (LK). The blood vessels listed are: median pallial artery (m.p.a.), common aortic trunk (c.a.t.) and (left and right) efferent ctenidial veins (e.c.v.).

The circulation in *Haliotis sp.*, based primarily on Crofts' account (1929), is outlined in Figure 1.1. Two atria (LA, RA) fill into a common ventricle (V), from which the blood leaves by the common aortic trunk, and is distributed to the organs. A connection between the cephalo-arterial sinus (CAS) and the cephalo-venous sinus (CVS) is reported in *H. ruber* (Russell and Evans, 1989) but has not been found in other *Haliotis* and is discussed further in chapters three and four. The mantle circulation appears to be primarily supplied by the small median pallial artery (m.p.a.) which originates directly from ventricle. The common aortic trunk (c.a.t.) is the major vessel which exits the ventricle and progresses anteriorly, giving off various arteries which supply most of the tissues of the animal, before widening into the CAS (Crofts, 1929; Russell and Evans, 1989).

The right kidney is perceived as the common venous drain, i.e. it forms a renal portal system in which the blood from the various tissues is collected here before progressing to the gills via the basibranchial sinus (Figure 1.1:gills, BBS) (Crofts, 1929; Russell and Evans, 1989).

Several studies on the circulatory physiology of *Haliotis sp.* have examined the blood pressure, haemodynamics, heart rate, and factors which affect these (Bourne, 1974; Bourne and Redmond, 1977a; 1977b; Jorgensen et al., 1984; Krajniak and Bourne, 1987; 1988; 1989; Russell and Evans, 1989; Taylor, 1993). A single study on *H. cracherodii* (Jorgensen et al., 1984) examined cardiac output, stroke volume, and tissue specific blood flow in resting animals. Clearly there is a need for more measurements of this kind, on more species, under a range of physiological states.

Jorgensen et al (1984), used a radioactive microsphere method to trace the distribution of the blood to the tissues and determine the cardiac output and stroke volume. The recent availability of fluorescent microspheres potentially makes such studies more accessible and, as described below, provided the primary incentive for the present investigation.

1.2.1 Clamping

The primary defence reaction in *Haliotis sp.* is to suddenly clamp forcefully to the substrate, thus thwarting potential predators. The consequences of the physiological state of clamping on the circulation in *Haliotis sp.* have not yet been examined with respect to the parameters of cardiac output, stroke volume, or tissue specific blood flow.

It has been proposed that during clamping in *H. corrugata*, blood in the adductor and foot muscles becomes isolated from the main circulation by closure of valves in the CAS and CVS (Figure 1.1) (Bourne and Redmond, 1977a; Crofts, 1929). These valves have not yet been found in *H. ruber* or in *H. kamtschatkana* and it has been suggested that the reduced blood flow to these muscles may simply result from occlusion of the LTS during clamping (Russell and Evans, 1989). Furthermore it has been proposed that during these periods blood may be redirected through a shunt from the CAS to the CVS in *H. ruber* (Russell and Evans, 1989).

Therefore, objectives of the present study were to determine changes in the blood flow distribution to the tissues during clamping, to elucidate whether a CAS to CVS shunt exists in *H. iris* during this state, and to infer which of the proposed mechanisms may be occurring.

1.2.2 Emersion

Because of their predominantly sub-tidal habitat, specimens of *H. iris* are seldom naturally exposed to periods of emersion. On the other hand during the artificial conditions of handling or freighting of abalone, emersion may be encountered for considerable periods of time (Baldwin et al., 1992; Wells and Baldwin, 1995). Whether changes occur in cardiac output function and blood distribution to the tissues, as a consequence of emersion is unknown but this seems likely to be the case.

The animals suffer internal hypoxia during emersion presumably due to problems with gas exchange and during this time the foot and adductor muscles rely heavily on anaerobic metabolism (Behrens et al., 2002; Wells and Baldwin, 1995) and bradycardia is a frequent response. Collapse of gills during emersion might be expected to interfere with cardiac functioning (Figure 1.1) and gas exchange.

Thus an objective of this research was to document and quantify blood flow changes in the tissues during emersion. The archaegastropod, *Acmea*, a limpet is reported to use the mantle to supplement gas exchange during aerial conditions (Kingston, 1968). The mantle appears engorged with blood and the ctenidia shrink, suggesting that changes in the distribution of blood flow occur.

While respirometry was not within the scope of this research project, perfusion of the ctenidia, epipodium and mantle, which are potential sites of increased oxygen diffusion in an aerial environment were of particular interest. Likewise changes in the perfusion of the potentially anaerobic foot and adductor muscles were of interest.

1.3 REGIONAL PERFUSION ANALYSIS USING MICROSPHERES

Microspheres have been used extensively to determine regional organ perfusion in vertebrates (Anon, 1999a; Barron et al., 1987; Cameron, 1974; Deveci and Eggington, 1999; Mulder et

al., 2001; Prinzen and Bassingthwaighte, 2000; Shultz et al., 1999; Van Oosterhout et al., 1995). The basic principle by which regional perfusion is examined using microspheres is briefly described as follows:

A sample of microspheres is introduced into arterial blood of the study animal, usually by injection in or near the heart, where they mix with the blood and are delivered to the various organs and tissues within the circulatory system. It is assumed that the microspheres are distributed uniformly across the cross-section of the vessel and that at each bifurcation are distributed in proportion to the flow to each branch (Prinzen and Bassingthwaighte, 2000). The microspheres lodge in the terminal vasculature of the tissues (termed entrapment) where they remain until their extraction (Anon, 1999a; Glenny et al., 1997; Prinzen and Bassingthwaighte, 2000). Thus the relative perfusion of any tissue is able to be calculated from the number of microspheres entrapped in that tissue (Anon, 1999a; Cameron, 1974; Shultz et al., 1999).

The method is based on earlier studies that employed radioactively labelled microspheres. Microspheres have since become available in a variety of materials, sizes and labels (Prinzen and Bassingthwaighte, 2000). The most suitable microspheres for regional perfusion experiments are fluorescently labelled uniform polystyrene beads, available in diameters of 10 or 15μm and labelled with fluorochromes of at least 10 colours (Anon, 1999a; Schimmel et al., 2001).

Fluorescent microspheres are superior to other labels in numerous aspects. They are relatively cheap to acquire, safe to handle, and have a good shelf life. They are of lower density than radioactive microspheres and thus more readily carried in the blood. They have high signal intensity and spectral overlap between the colours is limited, so protocols employing multiple serial injections can be used (Anon, 1999a; Prinzen and Bassingthwaighte, 2000; Schimmel et al., 2001).

The advantages of using more than one label simultaneously are that the regional perfusion under different conditions can be compared within the same animal and inferences can be drawn as to the changes in circulatory distribution which occur under the different conditions (Cameron, 1974; Glenny et al., 1997; Schimmel et al., 2001).

1.3.1 Extraction and quantification of microspheres

Tissues with entrapped microspheres are dissected from the animal and the microspheres can then be counted. While direct microscopical counting of microspheres in sections has been achieved for some tissues (Anon, 1999a), in most cases fluorescent microspheres must be isolated from the tissues before they can be quantified. This is achieved by digesting away the tissue matter, leaving behind the microspheres. The microsphere suspension is then filtered leaving the microspheres in the filter.

A more recently developed process, the sedimentation method has become the standard used to extract fluorescent microspheres from tissues (Anon, 1999a; Deveci and Eggington, 1999; Van Oosterhout et al., 1995).

1.3.2 The sedimentation method

The sedimentation method (Figure 1.2) relies on the principle that the microspheres sink (sediment) or may be spun down when in solutions of a lower specific gravity than the microspheres themselves. To achieve this, the tissues containing the microspheres are digested in an ethanolic solution rather than an aqueous solution. After centrifugation, the digestive fluid is aspirated off, leaving a pellet of microspheres, which after washing and buffering are ready to be assayed.

The filtration method is unlikely to be suitable for experiments on *H. iris*, as anecdotal evidence suggests that some tissues do not digest easily, leaving residues which would quickly clog the filter membranes. The sedimentation method is also faster, less labour intensive and also less expensive to apply. Results from the sedimentation method are reportedly equivalent to those obtained using the filtration method (Anon, 1999a; 1999c; Van Oosterhout et al., 1995).

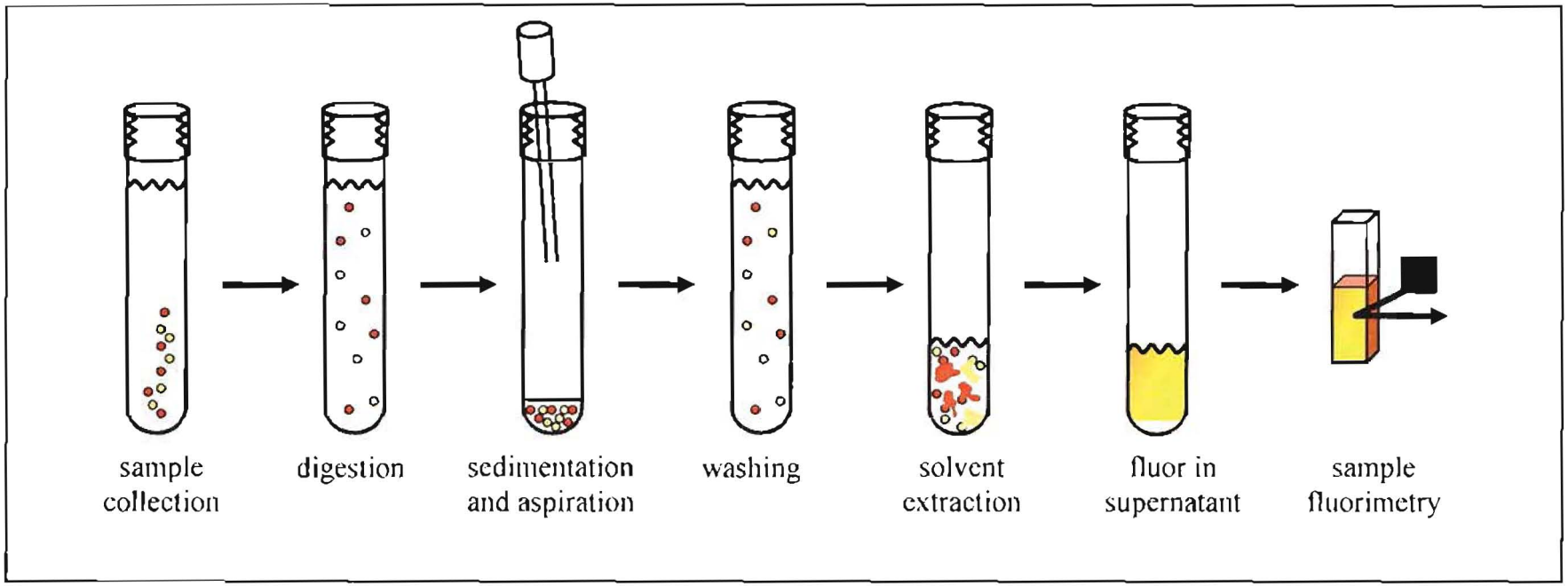


Figure 1.2. Outline of the sedimentation method of fluorescent microsphere extraction. The microspheres are represented as the small spheres in the original sample tissue. The tissue is digested and washed, with centrifugation, aspiration and pellet resuspension between each step. The microspheres are then dissolved in solvent and quantified using a fluorimeter. All solutions used during processing are of lower density than the microspheres, thus they sediment in the sample tubes.

1.3.3 Quantification of fluorescent microspheres

The microspheres contain a uniform amount of fluor, so that by assaying the quantity of fluor in the tissue samples we can determine the number of microspheres that were in each tissue sample. To assay the quantity of fluor in a sample the microspheres must be mixed with a known volume organic solvent, into which they will dissolve, thereby releasing the fluor into solution whereby it can be quantified.

1.3.4 Fluorimetry

Fluorescent substances absorb light from the UV-visible light spectrum, known as excitation, and rapidly emit photons of light at a different wavelength, known as emission, upon returning to their ground state (Anon, 1999a). The optimal excitation and emission wavelengths, and the difference between the two (known as a Stoke's shift), and the intensity of light released in relation to the amount of light used to excite the fluor (the quantum efficiency), is unique to each fluorescent substance.

A fluorimeter is used to measure the fluorescence intensity of the sample, and this is directly proportional to the number of fluorescent microspheres which were in the tissue. The concentration of microspheres in any given sample is able to be determined by comparing the samples against a standard curve of known concentrations of microspheres dissolved in solvent (Anon, 1999a).

1.3.5 Measurement of regional blood flow and cardiac output using fluorescent microspheres

The partitioning of fluorescent microspheres measures relative perfusion, the fraction of blood flow to each tissue from the injection site (cardiac output if injection is into the heart), of the sampled tissues. Changes in absolute blood flow, due to changes in cardiac output would not be detected. However, by including a reference withdrawal sample of blood at a known flow rate downstream of the injection site (a "dummy organ"), cardiac output can be calculated by the proportion of the injected microspheres distributed to the reference sample, and thus the relative flow to each tissue can be converted to actual flow units (ml/min·g tissue) (Anon, 1999a; 1999b; Deveci and Eggington, 1999; Jorgensen et al., 1984).

1.4 OBJECTIVES

The objectives of this study were as follows:

1) To adapt or develop a method for the determination of blood flow parameters, using fluorescent microspheres, in the black footed paua, *H. iris*, and evaluate its suitability, in terms of the kinds of information that may be obtained, and of the limitations of the method.
2) To use the method to document standard (resting) blood flow distribution and parameters in this species.
3) To examine the changes which occur to the blood flow and distribution under the conditions of clamping and of emersion, and integrate this into our current understanding of the circulatory regulation in this animal.
4) To evaluate whether the distribution of injected microspheres is consistent with the existence of previously hypothesised vascular connections and shunts in *H. iris*.

While fluorescent microspheres have been used extensively to examine circulatory systems in vertebrates, to my knowledge fluorescent microspheres have not been employed to examine the entire circulatory system in an invertebrate. There have been two earlier radioactive microsphere studies on invertebrates, one on another abalone (Jorgensen et al., 1984), and one on an amphibious crab (Taylor and Greenaway, 1984).

CHAPTER TWO

DEVELOPMENT OF THE METHOD FOR USING FLUORESCENT MICROSPHERES IN *HALIOTIS IRIS*

2.1 INTRODUCTION

The method of using fluorescent microspheres to examine regional perfusion is well established in vertebrates. This chapter reports the application of this method in *Haliotis iris*, and is divided into sections as follows:

2.2 The preparation of the animal for injection of fluorescent microspheres is reported, including shell cutting, cannulation procedures and heart rate monitoring.

2.3 The development of protocols for microsphere injection, reference blood flow sampling, and outlining their application to examine the circulation of *H. iris* under different physiological states.

2.4 The adaptation of the sedimentation method for the recovery of fluorescent microspheres from the organs of *H. iris*, later used in experiment chapters (Chapters three and four).

2.5 Quantification of fluorescent microspheres extracted from organ samples and calculations used to determine the relative perfusion and cardiac output in *H. iris*.

2.6 Evaluation of potential limitations in the application of the method used to determine regional perfusion and blood flow in *H. iris*.

2.2 ANIMAL PREPARATION

2.2.1 Animal capture and selection for experiments

Specimens of *H. iris* were collected under permit from Akaroa, Kaikoura and Motonau on the east coast of the South Island (New Zealand), and held in a 400 litre recirculating filtered seawater system at 15°C and a 12h:12h photoperiod. The holding tank was covered in black polyethylene to provide shelter from direct light and the animals fed on a combination of

artificial pellets (Adam and Amos™ AAFD), small amounts of dried ground *Gracilaria sp.*, and the film of algae present on the surfaces of the holding tanks.

Animals were allowed to acclimate at least 7 days following capture, and 2-3 days before experiments they were moved into a tank system with 700L of 15°C recirculating filtered seawater, and deprived of artificial food for 2-3 days.

Preparation for experiments required removal of specimens from the holding tanks, and to minimise time any animal was out of the recirculating seawater system, only one animal was prepared at a time. Animals were always detached from the substrate with a flat nylon spatula with smoothed edges, to prevent any damage to the tissues.
Selection of animals for experiments was random, but animals of an unhealthy appearance, or below 120mm in shell length, were not used. The animals in the experiments were up to about 170mm in shell length, and ranged from 160 to 550g in total weight.

2.2.2 Shell cutting

The animal was placed in a 10L container of continuously aerated seawater and taken to the laboratory where the shell cutting area was situated. The animal was removed from the container, placed on a 14mm diameter plastic petri dish to which it clamped firmly, and was thus easily tilted to assist shell cutting. At all stages of the preparation procedure the animal was regularly re-immersed in the container of seawater to prevent heat-stress and desiccation.

All cutting was done with a Dremel® MultiPro™ and diamond cutting wheel. The animal was regularly irrigated with seawater to prevent excessive shell dust and heat build-up, and extreme care was taken when cutting through the shell to prevent damage to the delicate tissues. Animals accidentally injured were not used in physiological experiments.

Two windows were cut in the shell of the animal, the first permitted access to the right efferent ctenidial vein and the aorta for later cannulation, and the second exposed the ventricle of the heart, allowing later optical monitoring of the heart rate (Figure 2.1).

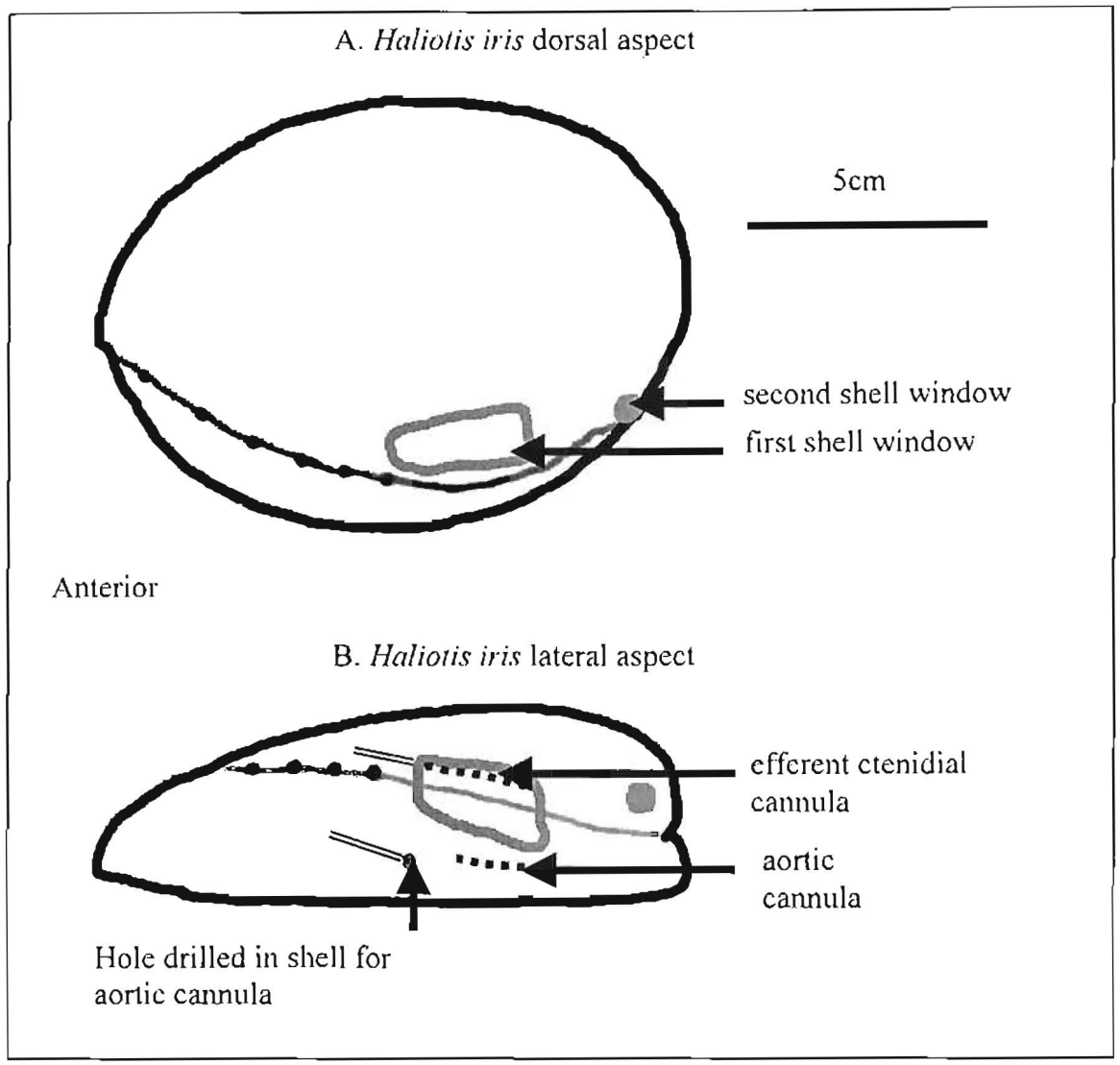


Figure 2.1. Diagram of shell cutting and cannulation sites in specimens of *H. iris* prepared for microsphere injection and reference withdrawal experiments. The first window was cut (diagram A) to permit access for cannulation, and the arrows on diagram B indicate where the two cannulae were inserted into the blood vessels, which are indicated by dashed lines. The drilled hole through which the aortic cannula was later inserted is labelled (diagram B), and the guides through which both cannulae were fed are indicated by the double lines (diagram B). The second window was cut and an optical probe later measured heart rate through this (diagram A).

The first window cut into the shell exposed most of the mucous gland, and gave access to two major blood vessels of interest (Figure 2.1). On the ventral side of the window beneath the mucous gland and left ctenidia, lay the aorta, the major efferent vessel from the heart. On the dorsal edge of window ran the right efferent ctenidial vein, from which the blood from the right gill drains into the right auricle (Chapter one).

The cut size of the first window depended on the animal size and was usually about 30mm wide and 20mm high in a specimen of about 150mm in shell length.
The second window was a smaller circular cut (approx. 8mm diameter) located directly over the ventricle (Figure 2.1), permitting later attachment of an optical probe to measure heart rate by ventricular movement.

During all cutting and preparation of the second window, damage to the extremely delicate pericardial and surrounding membranes was prevented by inserting a piece of Whatman® laboratory sealing film through first window (cut earlier) to cover the heart. The laboratory film was very soft and did not damage the tissues. Prior to the use of this protective measure, where even slight grazing of the pericardial membrane occurred, severe deterioration of the pericardium over the next 12hrs ensued, and in most cases the ventricle and atria were no longer contained effectively, inevitably resulting in death of the animal.

The inside edge of the second window was bevelled to remove sharp edges on which the tissues might otherwise have been damaged when the animal protruded its viscera, a phenomenon seen to occur during clamping, during which the viscera would presumably be pressed against the shell in a normal intact animal.

A 14mm diameter circular disc of clear copier transparency (EPM presentation products), was placed over the second window and sealed in place using hot-melt glue, preferred to other adhesives because of its suitability for filling gaps, and the cure speed and neutrality. The hot-melt glue was never allowed to contact the internal side of the window to prevent damage to the animal tissues and to the clarity of the transparency window on which the optical probe later relied for signal transmission.

Precautions against heat damage to the tissues were taken by keeping them at least 15mm from the gluing area by inserting a short piece of 10mm diameter silicone tubing (Masterflex®) inside the shell. The viscera were still protected by the sealing film, and the animal was immersed in seawater promptly after the second window was sealed.

A section of closed-cell foam (18x18x8mm) was bored with an 8mm diameter hole into which the probe later fitted closely, and hot glued to the shell, with the hole oriented directly over the second window.

Two grooves approximately 20mm long and 2-3mm deep were cut into the shell from the top and bottom anterior side of the first window, towards the anterior, into which two 20mm lengths of cannula tubing (OD:1.50mm; ID:1.00mm; Dural plastics and engineering, NSW) were set using Selleys® Supa glue Gel. These acted as guides through which the cannulae were later fed , and a small hole just anterior to the lower edge the window was drilled to later feed the aortic cannula through (Figure 2.1. B). The animals was placed back in the recirculating seawater system and allowed to rest for a few hours before the cannulation procedure.

2.2.3 Cannulation procedure

As was done for shell cutting, the animals were removed from the recirculating seawater system and placed on a 14mm Petri dish. During the cannulation procedure, two cannulae were inserted into the animal, the first (OD:0.8mm; ID:0.4mm; P.V.C.; Critchley Electrical Products, NSW) was inserted into the aorta and the second (OD:0.61mm; ID:0.28mm; polyethylene; Critchley Electrical Products, NSW) into the right efferent ctenidial vein.

The cannulation is described in detail as follows:

Two cannulae about 20cm long were prepared for insertion by trimming the end at an angle of approximately 35 degrees.

The first cannulation was into the common aortic trunk, which was located by gently lifting the mucous gland and left ctenidia. A section of this vessel 20-30mm long was clearly visible, originating posteriorly from the ventricle, running parallel to the ctenidia, and disappearing anteriorly from the surface where it continued, widening into the cephalo-arterial sinus (CAS).

A small puncture approximately 10mm posterior to where the aorta anteriorly leaves the surface was made into the vessel with a 25gauge hollow needle (Terumo®). The vessel immediately bled, and the cannula (OD:0.8mm; ID:0.4mm) was quickly inserted into the puncture, forming a seal which stopped the bleeding. The cannula was fed about 10mm towards the heart (posteriorly), thus insertion was retrograde with respect to the direction of

blood flow. Correct cannula placement was confirmed by a ready flow of blood through the cannula.

The other end of the cannula was fed through the hole drilled in the shell, and the cannula guide (Figure 2.1) to which it was secured by a small amount of Selleys® Supa glue, thus ensuring the cannula did not move in the vessel or attain angles which strained the insertion point.

A removable plug was made by adhering a small piece of closed cell foam to a crimped, wax plugged 25 gauge needle with luer fitting, and the needle inserted into end of the cannula to prevent bleeding. The closed cell foam, coloured to mark the vessel into which the cannula was inserted, kept the end of the cannula afloat, preventing it from getting caught under the animal.

The right efferent ctenidial vein is not always immediately obvious as it is not immediately on the surface of the viscera, thus often requiring location using a Doppler flow probe (Iowa Doppler products), which when touched gently against the tissue directly over the surface of the vein resulted in an audible signal of the blood passing through the vessel.

The cannulation procedure was essentially the same as described for the aorta. A small puncture was made approximately 30mm before the vein enters the right atrium, with a 27 gauge hypodermic needle, and the cannula (OD:0.61mm; ID:0.28mm) was immediately inserted and fed approximately 5mm towards the heart. Thus placement was orthograde with respect to the direction of blood flow. The cannula was bled briefly to ensure correct placement, fed through and fixed to the guiding tube with Selleys® Supa glue (Figure 2.1), and plugged with a 27 gauge needle prepared as for that of the aortic cannula.

The material of the cannula tubing used depended on the location of the cannula. The aortic cannula (OD:0.8mm; ID:0.4mm) used was made from P.V.C., as this is softer and conformed more readily to the angles required without damaging the insertion point, whereas the efferent ctenidial cannula was considerably thinner (OD:0.61mm; ID:0.28mm) and the polyethylene variety was used as it was slightly stiffer and more readily facilitated insertion and correct placement.

The diameter of the cannulae was determined by their requirements, and the aortic cannula was relatively large (OD:0.8mm; ID:0.4mm) as insertion was into a large vessel and it was required for blood withdrawal, whereas the efferent ctenidial cannula was smaller (OD:0.61mm; ID:0.28mm) as it was only used for injection and it was important to ensure it did not interfere with blood flow in the relatively small vessel into which it was inserted.

During initial cannulation trials a short length (~7mm) of 25gauge hypodermic needle was fitted to the end of the cannula and directly inserted about 5mm into the vessel without the need to create a puncture first. This method was not used in experiments as the implanted needle easily damaged the vessel if the animal or cannula moved.

After cannulation, the animals were individually placed into 2l plastic containers and returned to the recirculating seawater system where they were supplied with aerated filtered seawater. The heart rate monitor was fitted (described later) and the animals allowed to rest for 24 hours before any experimentation.

Animals were always used within 48 hours of cannulation as a precaution against infections or immune responses which could potentially affect their physiology and the patency of the cannulae. Cannulae implanted for several days (>72hrs) usually formed a plug, presumably of haemocytes, which was only sometimes able to be withdrawn by gentle vacuum and often occluded the cannulae.

2.2.4 Heart rate monitoring

Measurement of the heart rate during experiments was important to confirm that the heart was beating during injection and entrapment of the fluorescent microspheres, and to investigate how heart rate, a component required to calculate cardiac stroke volume, varied with experimental treatment.

A sealed optical probe which detected ventricular movement was manufactured and this fitted closely into the probe mount located over the ventricle (the second window described in Figure 2.1). The probe was connected to a 2-channel chart recorder (BBC) via an amplifier with which the gain could be adjusted, permitting recording of the probe signal. The placement of the probe could vary the signal considerably, and the required placement for

optimal detection of ventricular movement was established shortly after cannulation, recorded and later confirmed just prior to the experiment.

Correct placement was verified by correlating the detected signal with visually observed ventricular contraction. The probe was removed and later re-inserted into the animal, not less than one hour before the experiment, when it was confirmed that probe placement was producing a suitable signal.

Earlier attempts at heart rate monitoring used an impedance system consisting of two copper wires with the terminal insulation stripped, resting on the surface of the viscera just above the heart. The changes in impedance between the two electrodes, provided correctly placed, corresponded to the movements of the heart and were amplified and plotted on a chart recorder. The results were reasonable but the system was intolerant to movement by the animal and was too responsive to external noise, such as water movement or inducing the animal to clamp by hand. The optical heart rate monitoring system was very robust because of the encapsulated nature of the probe, which was able to be removed and repositioned conveniently.

2.3 MICROSPHERE INJECTION

2.3.1 Development of the injection protocol

The microspheres were injected into the circulation of the animal where they were distributed to the tissues in a pattern which reflected the partitioning of cardiac output (Chapter 1).

Initial trials of injection procedure involved direct injection of 100µl of stock solution into the animal ($1x10^5$ microspheres) using a 100µl glass syringe (SGE, Australia), as slowly and steadily as was possible by hand. Injection by hand cannot easily be done at a slow or constant rate and the injection was completed in approximately 30 seconds.
A constant rate of microsphere injection is important, as variations in the pressure of injection can locally disrupt the circulatory system, and some vessels may contract on experiencing significant pressure fluctuations (a possible pressure regulatory function), thus potentially occluding flow, in which case injected microspheres would not be mixed into the blood properly. Correct mixing of the microspheres with the blood is important in reference blood

flow sampling (described later), and requires a slow and constant rate of injection, as a rapid or bolus injection results in streaming, the displacement of blood with a "band" of microspheres which are not adequately mixed with blood by the time they are distributed to the reference sample or tissue (Anon, 1999a), an effect to which smaller animals with less total blood flow are presumably more vulnerable.

Slow and even injection was accomplished using a Sage syringe pump (Sage Instruments), but another problem was observed; when a slow rate of injection was used, the microspheres tended to settle out with gravity, leaving a relatively large number remaining in the syringe after injection. The manual for using fluorescent microspheres to measure regional organ perfusion (Anon, 1999a) recommends vortexing the microspheres in the event that they settle in the syringe, however this severely impaired steady delivery of microspheres into the circulation, furthermore it is nearly impossible to generate sufficient vortex in the narrow bore of a 250µl glass syringe. This problem was overcome by vertically mounting the sage pump, thereby ensuring any settlement of microspheres was in the direction of the outflow (needle end) thus maximising the number of injected microspheres.

To further ensure that the microspheres were introduced into the circulation at a slow and steady rate, the microspheres were diluted in an injectate, the preparation of which is described below.

2.3.2 Preparation of injectate

Yellow-green and orange fluorescent microspheres 15.0µm in diameter were supplied by Molecular Probes® Inc., each in 10ml sealed bottles at a density of $1x10^6$ per ml with 0.05% Tween®-20 as an anti-flocculent and 0.02% thimerosal as a bacteriostat. The microspheres were stored in the dark at 4°C and exposure to light was minimised to prevent photo-bleaching.

Injectate and later fluorimetry standards preparation required sample withdrawal and sub-dilution from the stock solution. Complete and uniform suspension of the microspheres in the stock solution ($1x10^6$ microspheres per ml) was ensured by vortexing thoroughly for at least 30 seconds, sonicating for 60 seconds, vortexing for another 30 seconds, before the required quantity was immediately withdrawn using a 100µl glass syringe (SGE, Australia). Sonication

was always kept under one minute as it is reported that if excessive, this can melt the microspheres (Anon, 1999a).

The injectate was prepared by diluting the withdrawn stock sample ($1x10^6$ microspheres per ml) in filtered Instant Ocean® (Aquarium systems) saline with Tween®-80 (Tween®-80 prevented microsphere aggregation and adhesion to surfaces), to produce a final injectate with a density of 400 microspheres per µl and 0.1% Tween-80® in saline of similar osmolality to seawater, which approaches the blood osmolality of *H. iris* (HH Taylor and NRL Ragg, personal communication).

The volume later injected was 250µl, thus 100,000 microspheres of each colour was administered into each animal. A total injection of 100,000 microspheres was used to minimise statistical errors, as recommended by Molecular Probes (Anon, 1999b) and the manual for using fluorescent microspheres to measure regional organ perfusion (Anon, 1999a).

All syringes were washed thoroughly after use to prevent cross contamination of stock solutions and prepared injectates.

This method of injectate preparation was used in the experiments later reported in chapters three and four, and the injection was administered using the following protocol.

2.3.3 Protocol for the injection of fluorescent microspheres into *H. iris*

Animals were prepared as described earlier in this chapter.

Prior to injection, the cannulae were checked for blockages, and as far as could be established correct placement, by withdrawing about 20µl of haemolymph from the efferent ctenidial cannula, and 100µl from the aortic cannula, using a 100µl SGE glass syringe. The blood from the efferent ctenidial cannula was discarded and the blood from aortic cannula was set aside to later flush the cannula after microsphere injection. Specimens with blocked or incorrectly placed cannulae were removed from the experiments.

The injectate was prepared, as described earlier, vortexed and sonicated, and a volume of 250µl (100,000 microspheres) drawn up into a 250µl SGE glass-syringe and the needle inserted into the right efferent ctenidial cannula. The syringe was placed in the vertically mounted syringe pump (Sage Instruments) and the injectate (250µl) was injected into the animal over 1.5min.

After microsphere injection the 100µl of blood withdrawn earlier was injected using the sage pump, to flush any remaining microspheres through the cannula.
The empty 250µl glass-syringe was filled with distilled water and discharged quickly into a sample tube and this was repeated once more, to establish how many microspheres remained in the syringe.

Both syringes were washed thoroughly before re-use to prevent cross contamination of samples.

2.3.4 Reference withdrawal sampling

The published reference withdrawal protocol involves withdrawal of a "reference sample" at an exact rate, starting at the same time as the microsphere injection and continuing for about 2 minutes after the injection has finished (Anon, 1999a; Anon, 1999b).

The method of reference withdrawal used in abalone required modification because abalone are much smaller than mammals, for which the technique was originally developed, and if too much blood was withdrawn in the reference sample, this could reduce blood flow to the organs and the blood volume of the animal. The main concern in using the constant withdrawal sampling method developed for vertebrates, in *H. iris*, was the observation that if the blood was sampled from the aorta at a rate exceeding the cardiac output of the heart, the aorta was drained of blood completely, and a vacuum immediately surrounding the end of the cannula in the vessel developed, preventing blood flow recovering to this vessel which was only restored when the vacuum was broken by injection of blood or saline into the cannula (unpublished observation).

This observation led to the development of a passive method of reference withdrawal, more suitable for use in *H. iris*, in which the reference sample was not extracted mechanically using

a vacuum, but instead the external end of the cannula was placed at a level below the animal, effectively creating a gentle siphon, the pressure head of which could be varied by increasing or decreasing the level of the cannula end relative to the animal.

I believe that this method is more appropriate for reference sampling from a small animal as the proportion of the cardiac output which is distributed to the reference cannula is proportional to the cardiac output of the heart; if the cardiac output decreases so does the pressure in the aorta and consequently less blood will be distributed to the reference cannula, thus greatly reducing the likelihood that too much blood will be sampled. The other advantage is that the pulsatile flow is similar of that to the tissues so this will not be a factor in the partitioning of the microspheres. The blood was collected directly into a sample tube and the amount of sample being "withdrawn" could be constantly monitored.

The flow rate of the reference sample was able to be calculated by dividing the volume of the reference sample by the time over which it was taken.

Unfortunately this method is not likely to be appropriate for reference sampling in vertebrates, because if the flow in the reference sample cannula becomes too low, as may happen during reduced cardiac output, then the blood is likely to clot at the internal intake of, or even in the cannula itself (Anon, 1999a). Clotting is a luxury of which abalone are incapable (Taylor et al., 1994; Taylor, 1993), thus it presents no issue in reference sampling from *H. iris*.

The method of passive reference withdrawal used in the experiment reported in chapter four is described below.

2.3.5 Protocol for passive reference withdrawal sampling in *H. iris*

The patency of the aortic cannula was verified as described in the injection protocol (100µl blood withdrawn), and furthermore it was checked that the blood would passively siphon when provided with a negative pressure head by lowering the end of the cannula below the level of the animal.

The reference withdrawal sampling was started at the same time as the microsphere injection "upstream" started by placing the end of the cannula below the level of the animal, and

collecting the blood in a 14ml polypropylene screw-cap sample tube. Passive flow was only attainable when the pressure head was negative.

The reference flow was stopped 5 minutes after it started, allowing ample time for the injected microspheres to pass the reference sampling point "downstream" in the circulation.

The calculations of cardiac output and stroke volume (CSV) are detailed later in this chapter.

2.3.6 Treatment groups and application of emersion and clamping

The treatments are described in more detail in later chapters.
The experiments reported in chapters three (emersion) and four (clamping) used two distinct labels, yellow-green and orange fluorescent microspheres; the first injection was always under "resting" conditions, and the second while the animal was either emersed (Chapter three) or clamping (Chapter four).

The physiological state of emersion provided unanticipated complications in measurement of the heart rate. After 24 hours of emersion, when the second injection was administered, the viscera were retracted away from the shell to the degree that the heart was no longer within detectable range of the optical probe, thus the heart rate was not able to be acquired successfully from these animals.

Controls were run for both emersion and clamping treatments, consisting of the second injection of orange microspheres at the same time delays as their treatment counterparts, but with the control animals in a "resting" state. The controls for both experiments are detailed further in the respective chapters.

2.3.7 Sacrifice

The animals were left for 10 minutes after the second injection to allow the microspheres to become entrapped in the tissues before they were sacrificed.
The aim was to despatch the animal as quickly as possible and with minimal disturbance, to prevent any alteration of physiological state.

A number of methods were tried but most were unsuitable, either because the animal did not appear to cease functioning quickly enough, as was observed when the head was severed, or the animal was placed in fresh water, very cold seawater, or in the freezer, or the specimen was rendered useless for dissection, as occurred when animals being frozen in liquid nitrogen disintegrated.

The most acceptable and gentle method of sacrifice was by placing the animal in a large volume (5L) of hot (50°C) seawater, similar to that used by Jorgensen et al (1984), who note that this method stopped the heart almost instantly and also caused the tissues to firm up thereby facilitating dissection.

The intact dead animals were placed individually into labelled plastic bags and kept at 4°C until dissection. All animals had total, shell and tissue weight recorded before dissection.

In trials animals were able to be dissected up to two days post mortem, provided they were kept refrigerated, beyond this time, or if the animals had been frozen, resolution of the different tissues during dissection became difficult.

2.4 MICROSPHERE EXTRACTION

2.4.1 Tissue sampling

Animals were placed on a tray for dissection and the tissues were separated from one another as in table 2.1, and placed into 14ml polypropylene screw-cap sample tubes. All sample tubes were weighed empty, and with tissues in them so that the weight of the samples could be calculated.

In earlier trials, samples of not more than 1 gram of tissue, and wherever possible whole organs, were dissected out and placed into sample tubes (Table 2.1). Due to difficulties in separating the digestive gland and gonad, and data from initial trials which revealed relatively low entrapment of microspheres in both these tissues (unpublished observation), they were later combined into one sample (Table 2.1)

A maximum of 1.5g of tissue was able to be put in the sample tubes to ensure an acceptable tissue:digestion fluid ratio in the later tissue digestion process. Organs exceeding 1.5g, such as the foot and adductor muscles, were sub-sampled (Table 2.1); pieces of about 1g in size were cut from the organ and the location of the sample was recorded as in the example in Figure 2.2.

Table 2.1. Tissue dissection and separation of samples

tissues sampled whole
heart
left kidney
left ctenidia
mucous gland
right ctenidia
right kidney

tissues sub-sampled
adductor muscle
digestive gland and gonad
epipodium
foot muscle
head
mantle
intestine and other viscera

In early trials, separate sub-samples of the foot were taken to examine regional heterogeneity of flood flow (Figure 2.2). Variability in microsphere distribution between sub-samples of the same organ was considerable, in some cases by orders of magnitude, when the data were expressed in mass specific terms (Figure 2.2 A).

Because of the spatial heterogeneity of the microsphere distribution (Figure 2.2), within the larger sub-sampled organs of an individual animal, it is highly unlikely that the sub-samples represent the actual proportion of microspheres entrapped in the whole organ. The estimation of the actual number of microspheres in an organ would be entirely dependent on the specific sub-sampled regions, for example if a large proportion of the injected microspheres was entrapped in a small area of the foot which was overlooked during sub-sampling, the total proportion of the flow received by the foot would be greatly underestimated. Data from sub-sampled organs were not be able to be used accurately for comparative purposes between animals because of variability of the exact size and location from which sub-samples were taken.

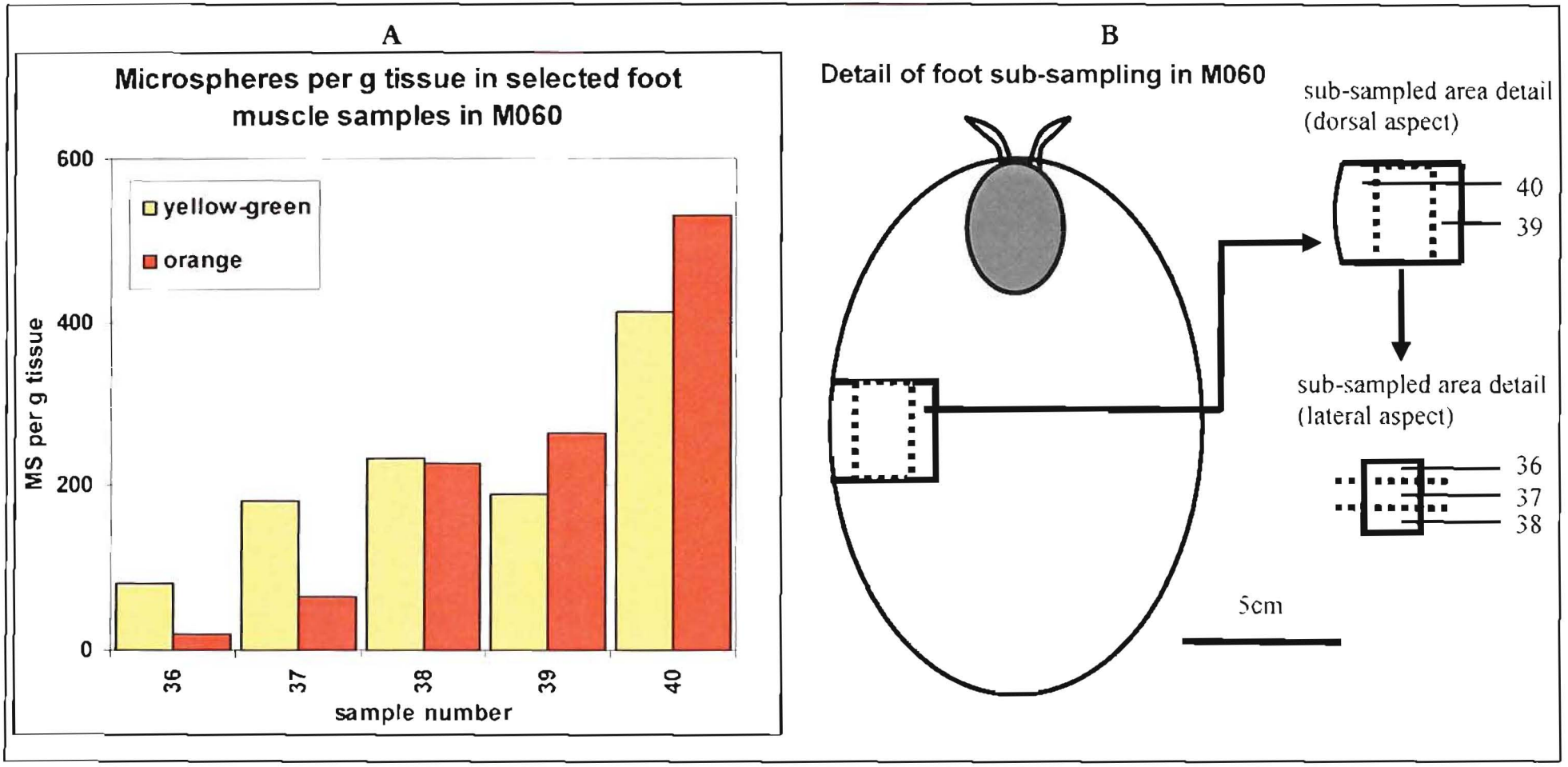


Figure 2.2. Heterogeneity of mass specific blood flow in five foot sub-samples of animal M060 as number of microspheres per g tissue (A). The yellow-green and orange microsphere distributions represent injections 10 minutes apart into a resting animal. The locations on the animal from which the five sub- samples (as numbered on A) were taken are shown in diagram B.

It was fundamental to this study that the changes in relative perfusion of significant organs, such as the foot, adductor and epipodium were able to be estimated accurately, compared to other animals under different physiological states at the time of injection, and that the total microsphere recovery from any animal was able to be estimated, as verification of the authenticity of the data. This method of sampling satisfied none of these criteria.

It was not practicable, both in terms of time and reagent costs, to sub-sample large organs such as the foot muscle in their entirety, or to later digest the organs whole. A different approach to sub-sampling of larger organs was used, that of homogenisation of the organ and sub-sampling of the homogenate.

The experiments detailed in chapters three and four used the following procedure for sub-sampling larger organs.

2.4.2 Homogenisation and sub-sampling

The tissues homogenised and sub-sampled were the same as those previously directly sub-sampled (Table 2.1): adductor muscle, digestive gland and gonad, epipodium, foot muscle, head, mantle, and intestine and other viscera. The organs were separated from one another and their individual weights recorded.

The addition of a known volume of distilled water was necessary to ensure that the sample did not heat up too much during homogenisation, as excessive heat could potentially damage the microspheres and tended to denature the proteins, thickening the sample and thus preventing the required homogeneity being attained. A tissue to water ratio of at least 1:2 was used, although some tissues, notably the foot and adductor muscle, required more water (water:tissue; 1:3) to ensure sufficient sub-sample homogeneity.

Homogenisation was done in two steps; first a relatively coarse homogenate consistency was attained using a Braun® Vario stick blender, then further homogenisation was done with an ultra-turrax® (Ika Labortechnik) until a uniform consistency with fine particulate size was attained. The homogenate was drawn up into a 10ml (Terumo®) standard syringe, and a sub-sample not exceeding 2.5ml dispensed into pre-weighed 14ml polypropylene screw-cap sample tubes.

As the weights of the sub-sampled organs and the exact quantities of water used during homogenisation were recorded, the actual amount of tissue (actual tissue weight) and proportion of the organ or tissue in any give sub-sample was able to be calculated, as detailed later in this chapter.

To ensure that the sub-sampled tissues were of a sufficient homogeneity, multiple sub-samples of homogenate were taken and compared, and it was later confirmed that the sample was uniform.

2.4.3 Sampling difficulties

Most organs were easily separated by dissection, with the exception of the digestive gland and gonad which as mentioned earlier was grouped together in one sample. The right kidney is an extensive organ which varies considerably in appearance between animals and even within the same animal (Crofts, 1929). Taking large samples of the right kidney proved exceedingly difficult as this organ is not very distinctly separated from several other organs, and while every effort was made, it was never able to be sampled as an organ in its entirety thus the samples taken were invariably small (about 0.2g) and probably quite variable. It is likely that both the intestine and other viscera, and the gonad and digestive gland sub-samples contain varying quantities of right kidney.

During initial trials only tissues of interest were sampled, but later experiments required the total recovery from the animal be estimated, and it became necessary to sample the animal in its entirety. The remaining tissue which was left behind after all other organs had been sampled appeared to consist primarily of intestine, and were grouped as one sample named "intestine and other viscera" (Table 2.1), homogenised and sub-sampled, ensuring complete sampling of the animals. The intestine and other viscera sample comprised a larger proportion of the animal weight during earlier experiments (average 5.7%)(Chapter three) than in later experiments (average 2.7%)(Chapter four) because of improvements made in the methods of dissection and separation.

The head sub-sample also consisted of multiple tissues, including the odontophore muscles and buccal mass, which were too small to warrant separate sampling, due to the errors which result from extremely small samples being taken, which are discussed later in this chapter.

2.4.4 The Sedimentation method and its adaptation for use in *H. iris*

The following section reports the sedimentation method used to extract the fluorescent microspheres with modifications which improved recovery from tissues of *H. iris*. Figure 2.3 is the sedimentation method described by Deveci and Eggington (1999) and was the initial method used to extract the microspheres from tissue in earlier trials.

The method is explained in the order of the steps in Figure 2.3.

1) Tissue sampling and placement into pre-weighed tubes was explained earlier in this chapter.
2) The weights of the tube with the sample were recorded, and the sample weight could be determined for calculations detailed later in this chapter.
3) 2M ethanolic KOH with 0.5% Tween®-80 was added to digest the tissues. Tween®-80 is a detergent, assisting digestion and maintaining dispersal of the microspheres in the solution.
4) Samples were left to digest in a heated shaking water bath. The fluorescent microspheres are safely resistant to alkaline hydrolysis at temperatures of up to 60 °C (Anon, 1999a). Samples were centrifuged to sediment microspheres and remaining tissue debris, and the supernatant was aspirated carefully to ensure microspheres were not lost by disturbing the pellet.
5) Distilled water was added to wash the pellet.
6) The pellet was resuspended to ensure efficient washing.
7) Ethanoic Tween® (0.5% Tween®-80 in 100% ethanol) was added to lower the specific gravity, ensuring microsphere sedimentation.
8) The sample was vortexed to ensure the ethanoic Tween® was mixed. Centrifugation and aspiration were as per step 4.
9) Phosphate buffer was added to neutralise the pellet as alkaline solutions reportedly quench fluorescence (Deveci and Eggington, 1999). Initial trials used 5ml of 100mM phosphate buffer (Deveci and Eggington, 1999), but as reported later in this chapter, this was reduced to 2ml of 200mM phosphate buffer.

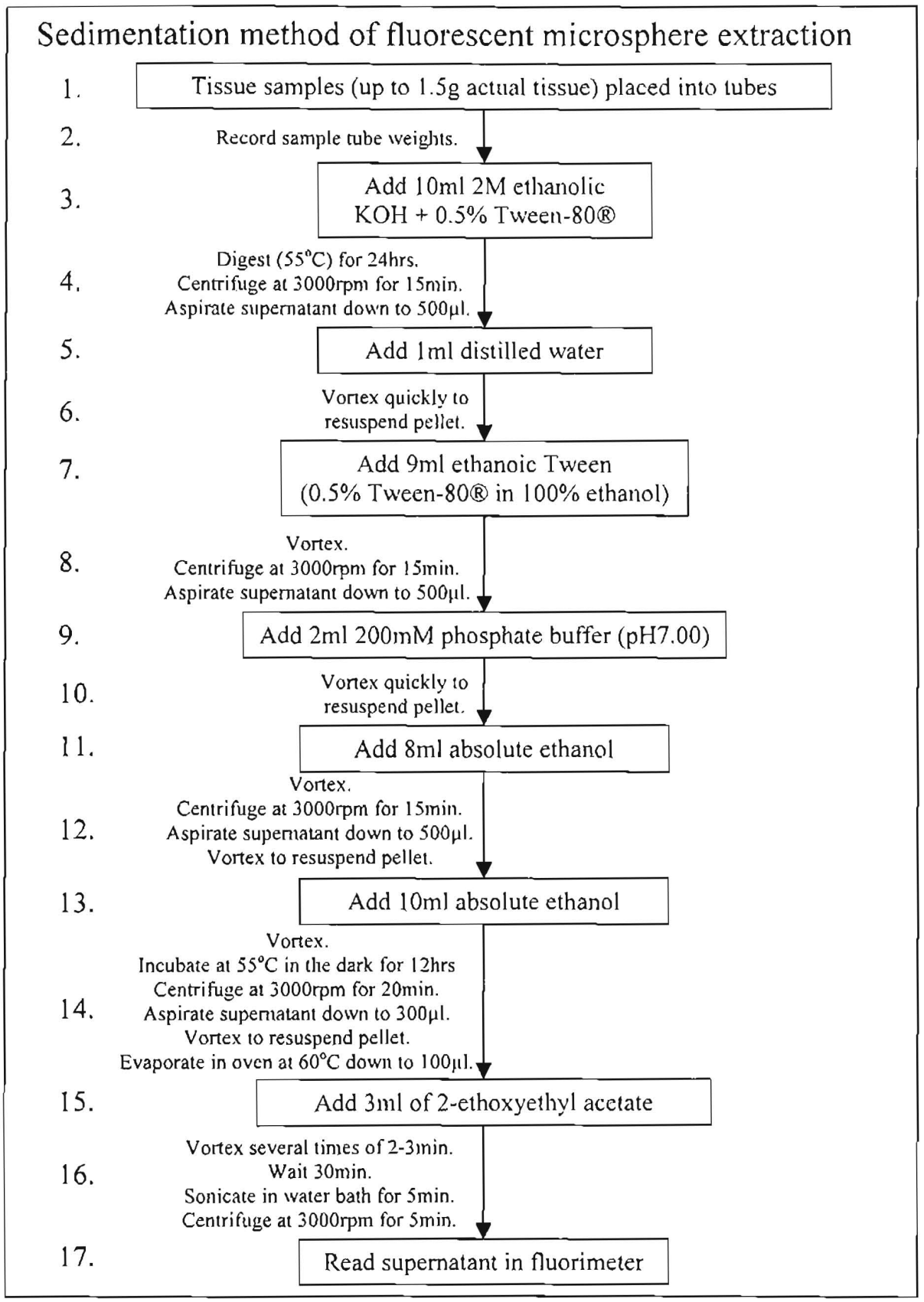


Figure 2.3. The sedimentation method of fluorescent microsphere extraction from tissue samples modified from Deveci and Eggington (1999). The alterations made to the original method are described in this chapter. This method was used in the experiments reported in chapters three and four.

10) The pellet was resuspended to ensure buffer penetration.

11) Absolute ethanol was added to reduce specific gravity, permitting microsphere sedimentation.

12) The sample was vortexed to ensure mixing of the ethanol. Centrifugation was as per step 4.

13) 10ml of absolute ethanol was added to the sample. This step was not in the original method used in earlier trials and as reported later in this chapter facilitated improved microsphere recovery from samples.

14) The samples were vortexed to ensure mixing of the ethanol and then incubated at 55°C for 12hrs, allowing the ethanol to dehydrate the pellet. Following dehydration, the sample was centrifuged for 20min, to ensure any residue was firmly sedimented. The supernatant was aspirated as low as possible, avoiding pellet disturbance. The remaining supernatant (about 300µl) was evaporated down to about 100µl in an oven, and care was taken not to dry the pellet completely as this may affect later extraction of microspheres from the tissue (Deveci and Eggington, 1999). The solvent used was 2-ethoxyethyl acetate (Cellosolve® acetate), which is mentioned later and differed from that used by Deveci and Eggington (1999).

15) An exact volume of solvent (3ml) was dispensed into each sample using an Eppendorf® multipipette® plus. It was critical that the solvent was dispensed accurately as this affects the concentration of the fluor for calculations detailed later.

16) The tubes were vortexed, left 30 minutes, and then sonicated for 5 minutes, to ensure the solvent dissolved all the microspheres. Centrifugation sedimented any remaining particulate matter which could otherwise later cause scattering in the fluorimeter.

17) The samples were taken to the fluorimeter and read within 24 hours, and the potential for the fluor to degrade over time will be reported later in this chapter.

2.4.5 Recovery of microspheres using the sedimentation method

Several specimens of *H. iris* were injected with both colours of fluorescent microspheres during initial trials to establish the best possible sampling method, (reported earlier in this chapter) and obtain preliminary information on the microsphere distribution in the circulatory system.

The sedimentation method was developed and is widely used to extract fluorescent microspheres from mammalian tissues, which characteristically digest very efficiently. The

tissues of the *H. iris* did not digest completely, often leaving a large pellet of residue before the final extraction. To the best of my knowledge, no other study has validated the process of extracting fluorescent microspheres from the tissues of an invertebrate using the sedimentation method, and it was necessary to determine the extraction efficiency of the process, to ensure the validity of experiments.

To this end a number of experiments were undertaken to validate and if necessary improve the method so that it could be used to meet the objectives of the research.

2.4.6 Recovery of spiked samples

Seawater samples and blank whole homogenized animal tissue sub-samples (about 1.5g each) were spiked and with a known quantity (3000 microspheres) of yellow-green and of orange microspheres and processed using the sedimentation method.

Un-spiked controls of the same sample type (seawater or whole animal homogenate) were also run. The tissue homogenate samples did not digest completely and an amount of indigestible matter formed a small dark coloured pellet after centrifugation. The seawater sample only formed a very small white pellet after centrifugation. The results are summarised in Table 2.2.

The spiked seawater samples had similar recoveries of both yellow-green and orange microspheres. In comparison to the spiked seawater samples a lower recovery of both microsphere colours was observed from the homogenate samples (Table 2.2). The orange microspheres appear to have a slightly higher recovery than the yellow-green in the homogenate, but the difference is not large.

The apparent recovery observed from the un-spiked samples represent background signal on the fluorimeter, the potential errors of which are discussed later in this chapter.

Because of the difference observed between microsphere recovery from the whole-animal homogenate samples and the seawater samples, and the differences between the pellets samples formed during the centrifugation steps of the sedimentation method, the possibility that the tissue somehow causes microsphere loss was suggested.

samples formed during the centrifugation steps of the sedimentation method, the possibility that the tissue somehow causes microsphere loss was suggested.

Table 2.2. Mean recovery of microspheres (MS) from samples spiked with yellow-green and orange microspheres (3000 microspheres of each colour), and un-spiked (control) samples of whole-animal homogenate and seawater. Values are means ± SEM of 3 replicates. The values in parenthesis are the percentage of the spike recovered.

spiked samples	seawater	homogenate
number of yellow-green MS in spiked sample	2858±31.0 (95±1.03%)	2413±39.2 (80±1.31%)
number of orange MS in spiked sample	2948±104.3 (98±3.48%)	2654±60.9 (89±3.49%)

control samples (un-spiked)	seawater	homogenate
number of yellow-green MS in blank sample	5±3.3	3±0.6
number of orange MS in blank sample	5±2.3	5±0.3

2.4.7 Investigation of potential sources of microsphere loss

In earlier trials of microsphere extraction from tissues of *H.* iris, it was observed that the digestive gland and gonad sub-sample digested very poorly, leaving a dark pellet which often failed to resuspend sufficiently during vortexing steps of the sedimentation method (Figure 2.3).

Because of the difficulty experienced resuspending pellets in samples of digestive gland and gonad it was considered possible that the Cellosolve® acetate might not fully penetrate the pellet during the solvent extraction (Figure 2.3, steps 15 and 16), failing to extract all the microspheres from the sample. An investigation into efficiency of recovery from the digestive gland and gonad sub-sample was conducted. Adductor muscle tissue was included as a comparison, as in initial trials it was observed to digest very well, leaving only a small white pellet.

in microsphere extraction efficiency. When considering samples with low microsphere recovery it is important to distinguish between these two sources of loss.

Causes of microsphere loss due to incorrect experimental technique were also examined in this investigation. The consequences of excessive aspiration (no supernatant left behind, care taken not to disturb pellet) and insufficient aspiration (1ml of supernatant always left behind) of spiked blanks where aspiration was required (Figure 2.3) were investigated. The effects of allowing the pellet to dry out completely during the final evaporation (Figure 2.3 step 14) in both adductor muscle and digestive gland and gonad sub-samples, which Deveci and Eggington (1999) caution may reduce solvent penetration, and the opposite, excess water (about 1ml) in the final solvent extraction step of spiked blanks (Figure 2.3 steps 15 and 16) were also investigated. The controls run in this experiment were not processed using the sedimentation method and were an estimation of recovery from a Cellosolve® acetate solvent blank, and directly spiking Cellosolve® acetate with microspheres.

All treatments were conducted in triplicate and all the samples were spiked with 3000 of both colours of microspheres (yellow-green and orange). Considerable difficulty was encountered with the digestive gland and gonad sub-samples during the pellet resuspension steps of the sedimentation method (Figure 2.3), as the viscous and sticky consistency of the pellet precluded adequate resuspension.

Further attempts to resuspend the pellet by attempting to break it up with a glass rod were unsuccessful as the sample simply adhered to the glass rod. The digestive gland and gonad samples were a consistently dark brown colour in both pellet and supernatant components, which despite lightening considerably as the sedimentation method progressed, caused a noticeable discolouration of the Cellosolve® acetate added in the final extraction step (Figure 2.3 steps 15 and 16), causing it to take on a golden colour.

The recoveries from the various treatments are presented in Figure 2.4.

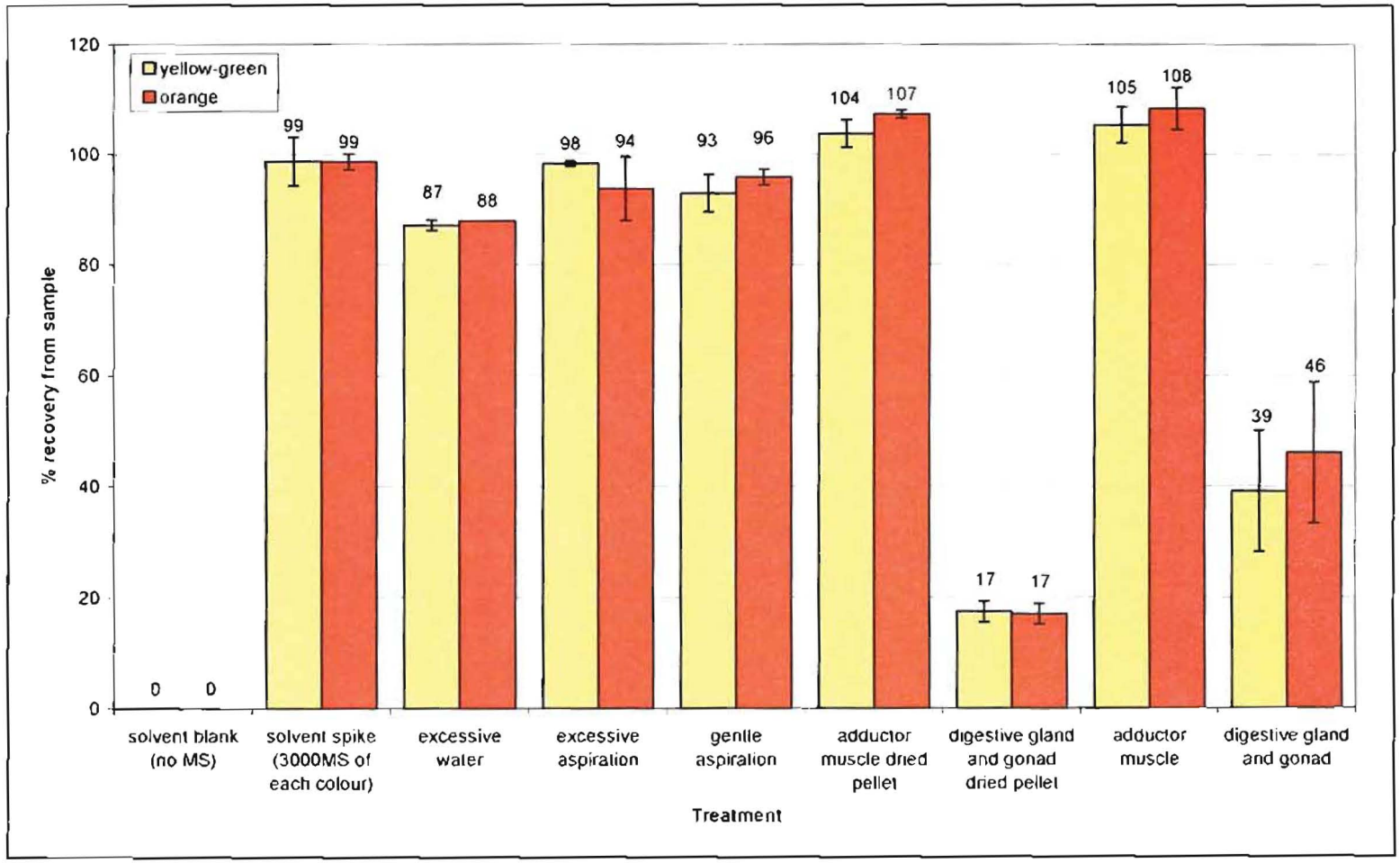


Figure 2.4. Average microsphere recovery from samples spiked with 3000 microspheres of each colour (yellow green and orange) the left bar is the yellow-green series and the right the orange series. Samples were subjected to various potential errors in experimental technique during processing using the sedimentation method. The potential errors examined are listed along the x-axis. Two controls were used in this experiment, the first was the solvent blank, with no microspheres, and the second the solvent spiked as per the samples (3000 microspheres of each colour). Spiked samples of two contrasting tissues, with respect to digestibility were also investigated. The adductor muscle characteristically digests completely whereas the digestive gland and gonad digests very poorly. The samples were all in triplicate (6 samples of each treatment, 3 for each microsphere colour) with the exception of the orange spike in the excessive water treatment which was only one sample and therefore the standard error is not supplied for this sample.

The microsphere recovery was very similar for the two colours of microspheres in all treatments (Figure 2.4), suggesting that no one colour is more likely to be lost than the other. Variable recovery between colours of the same treatment would suggest that the microspheres not of a sufficient similarity to be used for experiments where comparison of the two is required.

Low and variable recovery was obtained from the digestive gland and gonad sub-sample using the normal sedimentation method in both series (39±11% YG and 46±13% OR) (Figure 2.4). Very poor extraction was obtained from the digestive gland and gonad (17%) sample in which the pellet was allowed to dry out before the final solvent extraction (Figure 2.4), illustrating the importance of preventing this from happening to tissues in subsequent experiments.

The poor microsphere recovery from the spiked digestive gland and gonad sub-samples appeared to be primarily because of the impermeability of the pellet to the Cellosolve® acetate during the final extraction. This is evident from the dry pellet recovery which is even lower than the normal pellet, presumably as a consequence of it being even less penetrable to the solvent. The Cellosolve® acetate discolouration which occurred during microsphere extraction from the digestive gland and gonad sub-sample most likely has a detrimental effect on the fluorescence of the sample and must be considered as a source of error. However, this effect is probably comparatively minor compared to the very poor extraction efficiency from the pellet.

The adductor muscle had a very high recovery (>100%) regardless of whether the pellet was normal, or dried out prior to the final solvent extraction, or (Figure 2.4).

Of the potential experimental errors examined, only excessive water content in the final solvent extraction (about 87% recovery) caused a noticeable decrease in recovery compared with the control of the solvent spike (99%)(Figure 2.4). Excessive hydration can cause the Cellosolve® acetate to appear cloudy if the water is emulsified with the solvent during vortexing or sonicating (Figure 2.3), and this causes scattering of the light during later fluorimetry and a decreased optical clarity of the sample.

The solvent blank controls showed a nil recovery as expected, indicating that the lower end of the standard curve used to establish microsphere densities in the samples is fitted correctly, which will be mentioned later in this chapter.

Interestingly, excessive aspiration did not cause any loss (Figure 2.4), suggesting the microspheres are sedimenting properly and are probably firmly packed into the pellet, or the bottom of the sample tube during centrifugation. If the microspheres were loosely sitting on the top of the pellet after centrifugation they would more easily be aspirated (Figure 2.3) and a greater loss would be expected. The poor recovery of microspheres from the digestive gland and gonad sub-sample also supports this notion, as low penetrability of the pellet to Cellosolve® acetate probably prevented microsphere recovery, indicating the microspheres are in the pellet.

This experiment demonstrated that the recovery of microspheres using the sedimentation method is relatively robust to most of the potential errors in experimental technique investigated. In subsequent experiments care was always taken to ensure that samples never dried out before the final extraction step (Figure 2.3), as this could clearly affect extraction from some tissues.

The pellet from the digestive gland and gonad sub-sample was mentioned earlier as being of a consistency which made it considerably difficult to resuspend at the several steps where this was required (Figure 2.3). Thus it is likely that the undigested remains of the tissue are effectively "insulated" from the various steps of the sedimentation method, which are designed to facilitate later microsphere extraction.

Due to the low recovery and sample variability observed in the digestive gland and gonad it was impossible to apply a corrective factor to accurately calculate the actual number of microspheres trapped in this tissue. If the data from the digestive gland, and potentially other organs which do not digest well, was to be integrated into a model of the circulatory distribution in *H. iris*, a means of achieving a more consistent and improved recovery had to be developed.

The final evaporation step in the sedimentation method prior to the addition of Cellosolve® acetate (Figure 2.3 step 14) usually took several hours, during which some samples could

easily accidentally dry out, while others could retained an excessive amount of water. The earlier observation that excessive water during the final extraction caused a decrease in measured microsphere recovery (Figure 2.4) and an attempt to reduce the aqueous composition of the supernatant during evaporation prior to solvent extraction (Figure 2.3 step 14) prompted the use of half the volume of aqueous phosphate buffer at twice the strength in subsequent experiments, 2ml of 200mM buffer instead of 5ml of 100mM buffer (pH=7.00) (Figure 2.3 step 9) followed by 8ml of absolute ethanol instead of 4ml (Figure 2.3 step 11).

Deveci and Eggington (1999) note that microsphere loss during sample processing occurred when the aqueous phosphate buffer was added (Figure 2.3 step 9), and it did not seem unreasonable to reduce the aqueous composition of both the supernatant in this step and in the final evaporation step.

The low recovery from the digestive gland and gonad sub-samples, taken together with the apparent impermeability of the pellet to solutions used during the sedimentation method, suggested that the key to efficiently extracting microspheres from this tissue lay partly in using a more efficient digestion method.

The following experiment assessed microsphere recovery from all sampled tissues (Table 2.1), using double digestion (both 2M ethanolic KOH) the reduced volume of phosphate buffer prior to final evaporation (Figure 2.3 step 9) mentioned earlier, and the effect of disrupting the pellet to facilitate resuspension. Two alternative methods of digestion were investigated at the same time in adductor muscle and digestive gland and gonad sub-samples, two tissues of contrasting digestibility.

Tissue specific recovery and the effect of disrupting the pellet during the sedimentation method are reported separately, after the trial of alternative methods of digestion which follows.

2.4.8 Investigation of different sample digestion protocols

A constraint placed on all solutions used in the sedimentation method is that the specific gravity has to be lower than that of the microspheres, otherwise they will not sediment during centrifugation and will be lost during aspiration (Deveci and Eggington, 1999).

It was hypothesised that more completely digesting the sample tissues would result in improved microsphere extraction and less discolouration of the solvent during the final extraction (Figure 2.3).

The possibility that sub-sampled tissues contained too much added water to permit complete digestion, or that the digestive capacity of the solution was used up before digestion was complete, was considered by investigating the efficacy of a double standard digestion (2M ethanolic KOH). Two other methods of digestion using pancreatin, and nitric acid were also investigated.

Sub-sampled homogenates of adductor muscle and digestive gland and gonad (about 1.5g of each) were each spiked with 1500 yellow-green fluorescent microspheres and subjected to one of three digestive regimes; standard digestion with 10ml 2M ethanolic KOH, 5ml of pancreatin (a digestive enzyme) solution (0.22g pancreatin and 1.75g KOH in 100ml distilled water), or 3ml 69% nitric acid (concentrated). All nitric acid samples were incubated in a waterbath at 55°C, and the pancreatin samples at 37°C, for 24 hours, after which the samples from both treatments had 7ml of ethanoic Tween® (0.5% Tween®-80 in absolute ethanol) added, to lower the specific gravity of the solution thereby encouraging microsphere sedimentation, and the samples were spun at 3000rpm (1500g) for 15min.

The quantity of residue left after centrifugation indicated the efficiency of the digestion procedures. The pancreatin digested samples had a considerable amount of residue, more than from standard 2M ethanolic KOH digestion, whereas the nitric acid had almost completely digested all the tissue, leaving only a small residue. The pancreatin required a second digestion of 2M ethanolic KOH (24 hours) to further reduce the tissue residue.

The samples were subsequently processed using the rest of the sedimentation method (Figure 2.3). The microsphere recoveries from the different digestion methods are summarised in Figure 2.5.

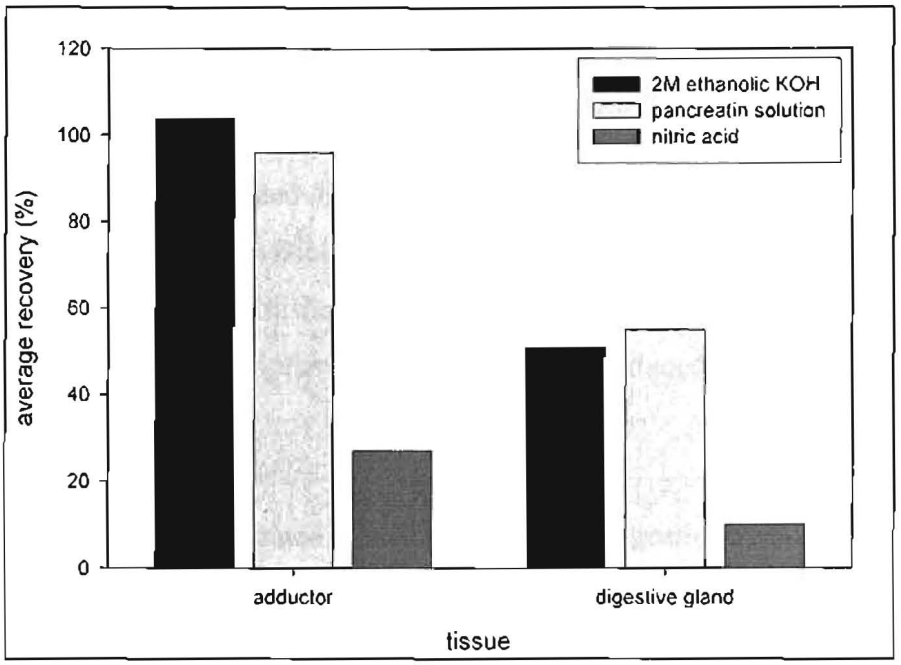


Figure 2.5. Percentage recovery of 1500 yellow-green microspheres from samples of homogenised adductor muscle (left series) and digestive gland and gonad (right series) subjected to three different digestion treatments: Double 2M ethanolic KOH (black bars), pancreatin solution followed by 2M ethanolic KOH (mid-grey bars), and nitric acid only (dark-grey bars).

Microspheres were recovered using both pancreatin, and double 2M ethanolic KOH digestions in both tissues (Figure 2.5), and a low recovery (less than 60%) was observed from the digestive gland and gonad (Figure 2.5). The microsphere recovery from adductor muscle was very good (about 100%) in both pancreatin and double 2M ethanolic KOH digestions (Figure 2.5). Nitric acid digestion clearly reduced the recovery of microspheres in both tissues investigated (less than 30%)(Figure 2.5).

The results of this experiment show no improvement in recovery in either tissue investigated from using pancreatin solution or a second digestion of 2M ethanolic KOH, compared to standard single 2M ethanolic KOH digestion (Figure 2.3) used in the previous experiment (Figure 2.4). The reason for the considerable reduction in recovery from the nitric acid digested samples (Figure 2.5) was not known. The loss could be attributable to chemical

damage of the microspheres themselves or may be due to their failure to sediment, and this was not investigated further.

It was suspected earlier that the relatively high water composition required of homogenised samples may have a dilution effect in the digestion step (Figure 2.3 step 4), effectively lowering the digestion fluid to tissue ratio. Consequently tissues which were difficult to digest may do so more poorly when homogenised with water. This did not appear to be the case, as after 24 hour digestion the aspirated digestion fluid was still highly caustic, and the pellet size following centrifugation did not visibly decrease following a second 2M ethanolic KOH digestion in this treatment (unpublished observation). Any effect homogenisation had on digestion was probably that of improving it because of the reduced particle size in these samples.

Of the digestion methods investigated, 2M ethanolic KOH digestion remained the most suitable and furthermore it appeared that a double digestion was redundant. The standard digestion method (2M ethanolic KOH with 0.5% Tween®-80) was used in all subsequent experiments.

It was concluded that the digestive gland and gonad of *H. iris* contains matter which is not able to be digested using 2M ethanolic KOH or pancreatin solutions.

The rest of the investigation described briefly earlier, examined microsphere recovery in all sample tissues and the effect of pellet manipulation during the sedimentation method.

2.4.9 Tissue specific recovery and the effect of pellet disruption

All tissues usually sampled in *H. iris* (Table 2.1) were dissected and if applicable, homogenized and sub-sampled, placed into sample tubes, and spiked with 1500 yellow-green microspheres. An additional two samples of both the adductor muscle and digestive gland and gonad homogenates were set aside and used for the purposes of investigating alternative methods of digestion as described earlier.

Two treatments were administered to all samples; the sedimentation method as normal (with the alteration to the phosphate buffer step mentioned earlier), and the same method but with

disruptive manipulation of the pellet, by trying to break it apart, to improve resuspension during steps of the method this was required (Figure 2.3).

Initially pellet disruption was done using the ultra-turrax® to try and homogenize the pellet and sedimentation solution. However when attempted, the pellet adhered to the tool, and the sample was lost. Subsequent attempts at manipulating the pellet were done using a glass stirring rod, and considerable difficulty was experienced with the pellets of many tissues, especially the digestive gland, epipodium, mucous gland and ctenidial samples, as the consistency of the pellet caused it to smear against the sides of the sample tube and stick to the glass rod, rather than simply breaking up and resuspending. This problem was most noticeable in steps of the method during which addition of aqueous solutions occurred (Figure 2.3 steps 5 and 9), and caused some sample loss. The results for the experiment are detailed in Figure 2.6.

The method of pellet manipulation appeared to improve recovery of microspheres from samples which normally had comparatively low recoveries, such as the mucous gland (80% normal; 92% disrupted), right kidney (92% normal; 99% disrupted) and most notably the digestive gland and gonad sample (51% normal; 91% disrupted) (Figure 2.6).

In most tissues where pellet disruption treatment was applied, an apparent loss of microspheres is evident. Recovery after pellet disruption in the left kidney, right ctenidia, intestine and other viscera, epipodium, and adductor samples, is somewhat lower compared with the normal treatment (Figure 2.6).

These losses are likely due to the physical loss of sample which occurred as a result of the it adhering to the glass rod used for pellet disruption, as mentioned earlier. Where improvement appears to have occurred, a loss of microspheres due to a loss of sample more than likely also occurred, but was obscured by the greater recovery produced by promoting better extraction. The disruption of the pellet produced estimated recoveries in excess of 90% for all tissues, but also appears to have caused losses in several tissues. The loss of sample which occurred during the pellet manipulation is a serious problem, as it cannot be predicted or corrected for with certainty.

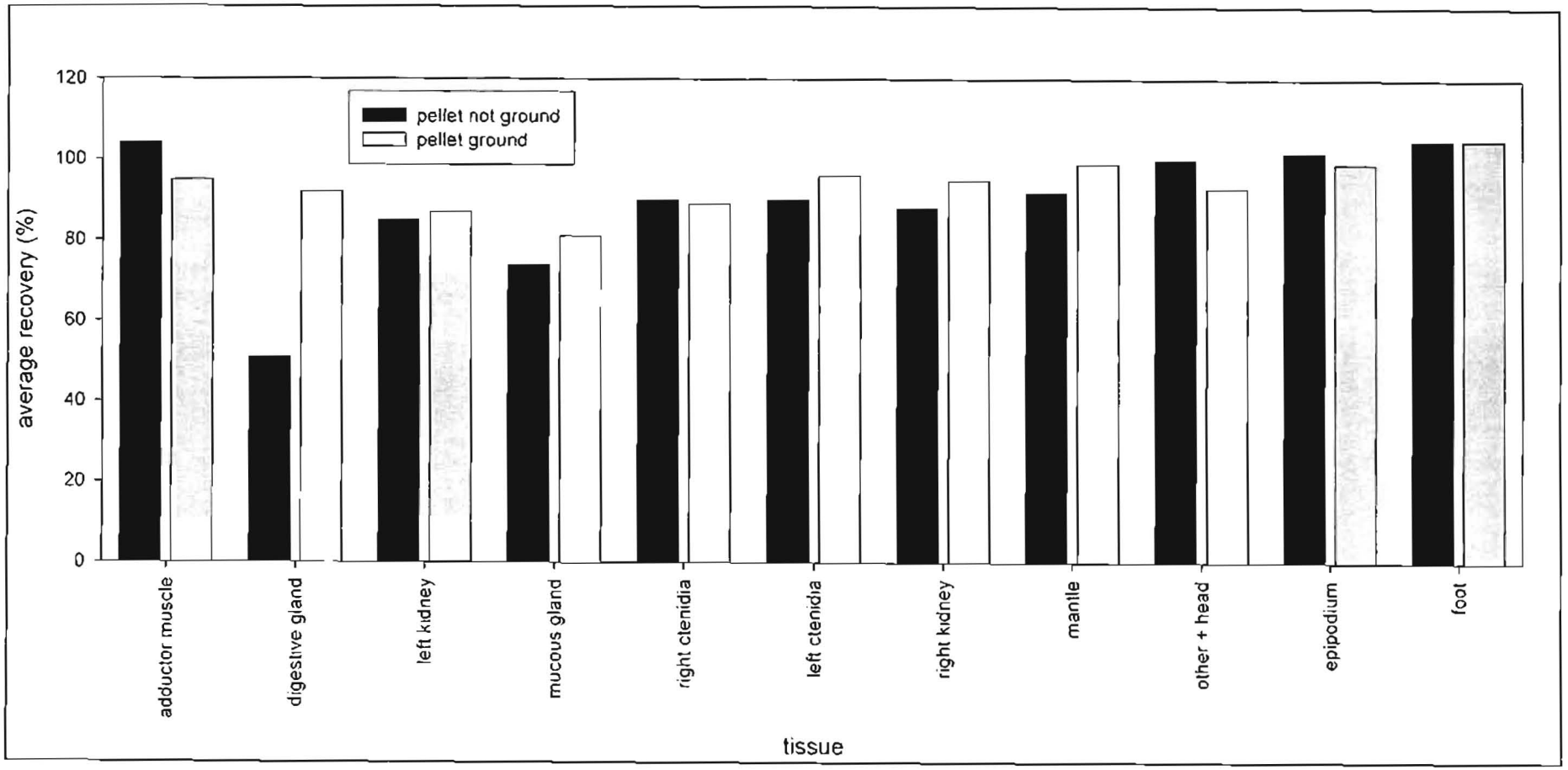


Figure 2.6. Percentage recovery of 1500 yellow-green microspheres from tissues using the normal sedimentation method (black bars), and an alteration involving mechanical disruption of the pellet during steps where resuspension was required (grey bars). The normal treatment for the adductor and digestive gland and gonad sub-samples are the same results as reported in Figure 2.5.

Manipulation of the pellet can only be used with certainty if sample loss is minimised. As mentioned earlier, most of the sample loss occurred after the two steps in which aqueous solutions had been added to the sample tube (Figure 2.3), as the pellets are least conducive to manipulation at these steps. It was suspected that a partially aqueous composition of the pellets causes the undigested matter to aggregate and prevents both manipulation without sample loss, and adequate resuspension.

2.4.10 Modifications to the sedimentation method to facilitate pellet resuspension and solvent penetration

The potential of water to interfere with solvent extraction efficiency has been alluded to earlier, as trial investigation results (Figure 2.4) suggested microsphere recovery was reduced when water was present in the solvent extraction process (step 13, Figure 2.3). In the previous investigation of pellet disruption water composition of the pellets appeared to cause the problems encountered in resuspending them.

The amount of water used in the sedimentation method had already been reduced with the modification of the buffering step, but too much water was likely still present in the pellet at the final extraction step. Efficient solvent penetration of a pellet with a relatively high aqueous composition will not occur as Cellosolve® acetate is hydrophobic, whereas a pellet with primarily ethanolic composition would presumably be more penetrable by the solvent. Cellosolve® acetate penetration of residual indigestible matter, whether in pellet form or suspension, is critical as the solvent must come in contact with the microspheres to dissolve them and release the fluor into solution for later fluorimetry.

Complete dehydration of the pellet might facilitate its disruption without loss of sample and permit resuspension, and an extra dehydrating step, the addition 10ml of absolute ethanol, was inserted between steps 12 and 13 (Figure 2.3) of the method. The ethanol was added to the samples, which were then left for 12 hours at 55°C to allow the absolute ethanol to penetrate and dehydrate the pellet.

The dehydrated pellets became solid and brittle and were able to be broken up mechanically without any apparent sample adhesion to the tool, thus minimising sample loss. Pellets were ground to a fine consistency against the base of the sample tube using a specially

manufactured acrylic tool, and care was taken to ensure that there was no visible loss of sample or cross contamination between samples, by ensuring that the tool was double rinsed in absolute ethanol and wiped between each sample.

This method of sample dehydration prior to solvent extraction was used in all later experiments and recovery estimates from whole animals were good, at around 90% (Chapters three and four). The extra dehydration and grinding step acted as a thorough final wash for the sample, thereby preventing any later discolouration of the Cellosolve® acetate which had been observed in previous experiments.

2.4.11 Microsphere extraction using Cellosolve® acetate

The samples were dried to a paste-like consistency with care taken to ensure that they were did not dry out completely. Exactly 3ml of Cellosolve® acetate (2-ethoxyethyl acetate) was dispensed accurately into each sample tube using an Eppendorf® multipipette® plus, and subsequent vortexing and sonicating ensured efficient microsphere extraction (Figure 2.3).

Cellosolve® acetate consistently appears in the literature (Anon, 1999a), and was recommended by the supplier of the fluorescent microspheres, Molecular Probes® Inc., as the most suitable solvent for their extraction (Anon, 1999b; Anon, 1999c).

The volume of solvent used to dissolve the microspheres is critical for the accuracy of later measurements, as too much solvent will underestimate the number of microspheres in the sample and too little will cause the opposite. The volume used (3ml) was chosen because it was easily dispensed accurately, was within the capacity of the fluorimetry cuvettes used later, and later resulted in measurements which were within the working range of the fluorimeter. Solvent extracted samples were sealed tightly, as evaporation could potentially alter measured concentration, and Cellosolve® acetate is a health hazard.

The modifications to the sedimentation method permitted adequate and consistent recovery of microspheres from all tissues in *H. iris*. The sedimentation method, as has been mentioned earlier, is designed for use with mostly vertebrate muscle tissue which reportedly digests very well (Deveci and Eggington, 1999) and the problems encountered with low recoveries using the standard method are probably specific to *H. iris*.

2.5 FLUORIMETRY

2.5.1 Sample fluorescence and reading

Samples of Cellosolve® acetate containing fluor extracted from fluorescent microspheres using the sedimentation method detailed earlier in this chapter were read in a fluorimeter. Earlier test samples were read by a Shimadzu® Spectrofluorometric Detector (RF-551 PC), however this was a somewhat dated instrument and was not capable of high resolution required for accurate measurement of the samples. All results reported in this and subsequent chapters, were read in 10mm path fluorometric glass cuvettes by a Varian® (Cary Eclipse) fluorescence spectrophotometer, a machine with extremely high sensitivity and resolution.

The published spectra for the optimal excitation and emission wavelengths of yellow-green fluorescent microspheres is 495nm (excitation) and 506nm (emission), and orange fluorescent microspheres 534nm (excitation) and 552nm (emission) in Cellosolve® acetate (Anon, 1999a). To confirm that the published excitation and emission optima were the same as those in the samples, a 3-dimensional scan of a sample containing both yellow green and orange fluorescent microspheres (2000 of each) was read and the data are represented both 2-dimensionally and 3-dimensionally in Figure 2.7.

All samples were excited and read at these exact optima at an averaging time of 0.5 seconds per sample. The emission slit-width on the machine was able to be adjusted and was set at the default of 5nm and if a sample was of a very high fluorescence beyond the reading capabilities of the detector, the emission slit width was decreased to 2.5nm and the sample successfully re-read. Each sample was excited and read at the excitation and emission optima of the yellow-green and orange fluors separately and in succession.

Both graphs (A and B) in Figure 2.7 show the same data from a different aspect. The contour plot (A) shows the exact position of the optima in the spectra, marked as bisections and the 3-dimensional plot (B) graphically demonstrates the magnitude of the optima (in a.u.). The emission peak of the yellow-green fluor (140a.u.) is nearly double that of the orange fluor (80a.u.) indicating a nearly double quantum efficiency of this dye (Figure 2.7 B).

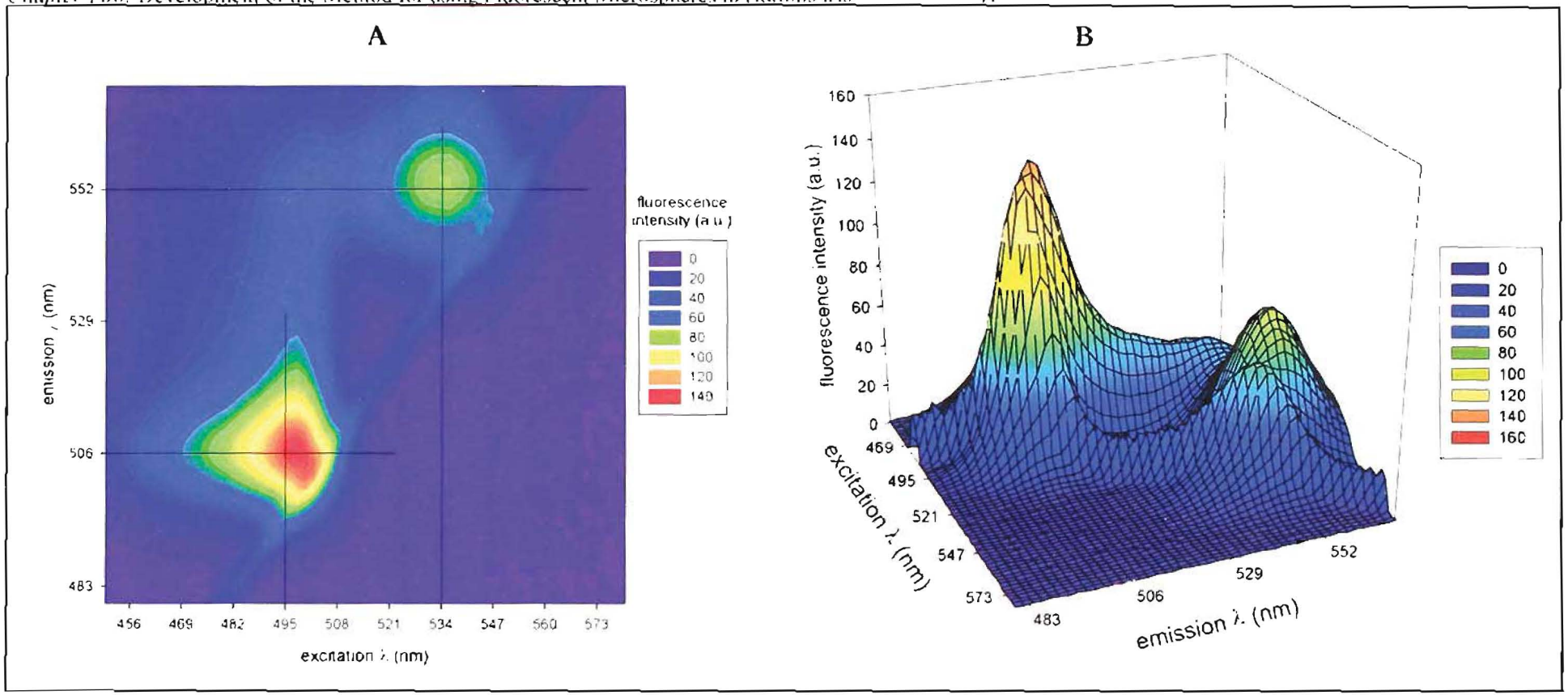


Figure 2.7. The excitation and emission optima of the fluorescent dyes of 1000 yellow-green and 1000 orange fluorescent microspheres extracted in Cellosolve® acetate, as determined by a 3-dimensional scan ranging from excitation wavelengths of 450nm to 580nm (in 2nm increments) and reading emission wavelength ranging from 480nm to 570nm. The excitation and emission optima (yellow-green 495mn, 506nm; orange 534nm, 552nm) are marked as lines on the 2-dimensional graph (A) and are clearly visible as peaks on the 3-dimensional graph (B). The yellow-green dye clearly has a greater quantum efficiency than the orange dye, as is seen by the greater fluorescence intensity. A relatively faint line of scattered light is visible as a straight line where Y=X. The Z axis units are in arbitrary units (a.u.). The lack of spillover between the two fluors is clearly seen by the distinction of the two optima (B).

The graphs show that the published optima for both yellow-green and orange fluors correspond to the observed optima, and also confirm that the experimental samples were being excited and read at the correct optima. The distinctly separate emission optima of the two dyes (Figure 2.7 B) confirm that a spillover correction does not need to be applied to samples, and is consistent with statements in the manual for using fluorescent microspheres to measure regional organ perfusion (Anon, 1999a) and a technical bulletin (Anon, 1999b) from the microsphere supplier, Molecular Probes® Inc., that correction is unnecessary between these two colours as spillover is non-existent.

The observed lack of scatter in the standards (Figure 2.7) and a visual inspection of each sample for optical clarity before reading ensured that this not a likely source of error.

2.5.2 Sample fluorescence degradation

The potential for the fluorescence intensity of a sample to change over time is a potential source of error, as the fluor is reportedly much more sensitive to degradation in the free state (Anon, 1999a). Deveci and Eggington (1999) suggest that the samples should be read within an hour of microsphere extraction, and report decreases in fluorescence intensities of between 1-5% percent (depending on the fluor) within an hour (Deveci and Eggington, 1999).

This could have been a problem for the present study because the large number of samples processed often took several hours to complete readings from. The manual for using fluorescent microspheres to measure regional organ perfusion (1999a) recommends that samples be read within 1-2 weeks of preparation and trial samples were checked by reading within an hour of preparation, re-reading 2 hours later, and then 72 hours later. No measurable deterioration was observed between any of the re-readings and it was assumed that provided samples are stored properly (4°C, dark) short-term deterioration is negligible. All subsequent experimental samples were read within 24 hours of preparation and were stored in the dark at all times prior to fluorimetry.

2.5.3 Cuvette variation

Variations between cuvettes can affect readings (Anon, 1999a), but is usually consistent and can thus be corrected for. To ensure that cuvettes gave uniform readings across samples

several samples were read in all cuvettes. There was no consistent variation detected, and provided the cuvettes were kept clean and undamaged, this was not considered as a source of error. All subsequent samples were read in individual cuvettes.
Cuvettes were rinsed with absolute ethanol and dried between sample readings to prevent cross contamination between samples.

2.5.4 Fluorimeter sensitivity and standards preparation

Initial checks were performed on both colours of microspheres by dissolving 10,000 microspheres in Cellosolve® acetate and sub-diluting to create a set of standards ranging from less than 10 to 10,000 microspheres per ml.

Sample fluorescence, measured in arbitrary units (a.u.), was linearly proportional to the density of microspheres dissolved in the sample up to the measured density of 10,000 microspheres per ml. However samples containing more than 2500 yellow-green microspheres or more than 5000 orange microspheres were outside the default detection range of the fluorimeter, thus as mentioned earlier the emission slit width of 5 nanometres was reduced to 2.5nm. Because of the lower quantum efficiency of the orange fluor, samples measured for orange fluorescence only required a reduction in emission slit width when the orange fluor density was above 5000 microsphere per ml.

Calibration curves were fitted for microsphere fluorescence, in arbitrary units, versus microsphere density, in microspheres per ml solvent as follows:

For yellow green standards at slit emission slit widths of 5.0 nanometres

$$y = 0.2390x_d + 0.303 \qquad \text{with } R^2 = 1.000$$

where y = the fluorescence intensity of the sample in arbitrary units (a.u.)
and x_d = the number of dissolved microspheres per ml of solvent

For yellow green standards at emission slit widths of 2.5 nanometres

$$y = 0.0443x_d + 0.0515 \qquad \text{with } R^2 = 0.998$$

For orange standards at emission slit widths of 5.0 nanometres

$$y = 0.1654x_d + 0.215 \qquad \text{with } R^2 = 1.000$$

For orange standards at emission slit widths of 2.5 nanometres

$$y = 0.0309x_d + 0.067 \qquad \text{with } R^2 = 1.000$$

The fluorescence blank used was Cellosolve® acetate without any microspheres and corresponded closely to the y axis intercept of the above equations. Samples of blank tissues without microspheres processed with the sedimentation method produced readings identical to the solvent blank readings, thus discounting the possibility that any autofluorescent components remained.

2.5.5 Calculation of microsphere density from sample fluorescence

The number of fluorescent microspheres extracted from a sample corresponded to the fluorescence of the sample at the specific optima mentioned earlier, and the concentration of dissolved microspheres was calculated by comparing the measured fluorescence against the calibration curves of known densities established earlier.

rearranging the formula for the linear calibration curves

$$y = mx_d + c$$

to make the unknown, x, the subject of the equation gives

$$x_d = \frac{(y - c)}{m}$$

Having determined the concentration of microspheres dissolved in the solvent the number of microspheres in any given sample x_s, was determined as follows:

$$x_s = x_d \times v$$

where v = 3ml solvent used to extract the microspheres in all experiments

Using the above formula, the number of microspheres in any of the organs sampled whole (Table 2.1) could be determined, and as the weight of the tube contents (w_{sample}) was known, the data could be expressed mass specifically (microspheres per g tissue). The number of microspheres in sub-sampled organ samples was only a fraction of the number in the whole

organ, and the number in the whole organ given the number in a sub-sample, was determined as follows:

2.5.6 Calculation of microspheres in whole organs from a sub-sample

The tissue proportion of the sub-sampled homogenate, p_{sample}, was needed to determine how much of the sample weight was actual tissue. This was calculated as follows:

$$p_{sample} = \frac{w_{organ}}{w_{water}}$$

where w_{organ} = the weight of the organ or whole tissue, in grams
and w_{water} = the amount of distilled water added, in millilitres
where 1.0ml distilled water = 1.0g

The actual tissue weight, w_{actual} in grams, in the sample was determined as follows:

$$w_{actual} = w_{sample} \times p_{sample}$$

The proportion of the sub-sample that the organ represented, p_{organ}, was calculated:

$$p_{organ} = \frac{w_{actual}}{w_{organ}}$$

where w_{organ} is the weight of the entire organ or tissue in grams

The weight specific microsphere content x_{sw}, and the number of microspheres in the whole organ, x_{organ}, could then be calculated as follows:

$$x_{sw} = \frac{x_s}{W_{actual}}$$

and :

$$x_{organ} = x_{sw} \times W_{organ}$$

2.5.7 Total recovery from the whole animal

The total number of microspheres recovered from the animal, x_{total} was simply estimated as the sum of the total number of microspheres recovered from all organs of the animal:

$$x_{total} = \sum x_s$$

This provided a verification of the results from each animal, and low recovery usually meant that the microspheres had not correctly entered the circulation, and this possibility is detailed later in this chapter.

The results for every sample were expressed as a proportion of the total number of microspheres recovered from the animal, thereby permitting better comparison of the data between sequential injections and between animals, by eliminating the variation caused by the assuming 100% delivery and recovery of the label.

2.5.8 Calculation of relative perfusion

The blood flow to each organ, as a percentage of cardiac output ($\%Q$), was simply estimated as a percentage of the total number of microspheres recovered in the animal (x_{total}). Expressing the data in terms of recovery accounted rather than in terms of the theoretical total number injected, ensured that if one label was recovered in lower quantities than the other, then the actual partitioning of cardiac output would still be comparable between the labels.

The data were also calculated as mass-specific percentages of cardiac output per organ, Q_{sw}, thus the samples could be compared to other samples of vastly different weights more easily. For example the percentage of cardiac output the foot receives was higher than any other

organ (about 40%)(Chapters three and four), however the percentage cardiac output per g tissue was one of the lowest of all the organs (about 0.5%).

The calculation method for the percentage of cardiac output ($\%Q$), and the mass specific percentage of cardiac output ($\%Q_{sw}$), received by any given organ was as follows:

$$\%Q = \frac{x_{organ}}{x_{total}} \times 100$$

and :

$$\%Q_{sw} = \frac{x_{sw}}{x_{total}} \times 100$$

2.5.9 Reference withdrawal calculations

The heart rate of the animal, Hf (beats per minute), and the average blood flow in the reference sample (f_{rf}) was calculated, and the number of microspheres in the reference sample x_{rf}, and the total number of microspheres recovered from the animal x_{total}, was calculated as described earlier.

The calculation for the absolute cardiac output, Q (ml/min), and cardiac stroke volume, CSV (ml), of the animal, based on the number of microspheres in the reference sample of known flow, is as follows:

$$Q = \frac{x_{total}}{x_{rf}} \times f_{rf}$$

and :

$$\mathrm{SV} = \frac{Q}{Hf}$$

Reference sampling data estimating Q and CSV are reported in chapter four.

2.6 POTENTIAL PROBLEMS USING FLUORESCENT MICROSPHERES

2.6.1 Potential sources of error

There are a number of potential sources of error which could occur in the application of a fluorescent microsphere method to *H. iris*. So far this chapter has reported the resolution of problems relating to microsphere extraction efficiency from the tissues using the sedimentation method, and investigated the potential problems of tissue autofluorescence, sample fluorescence degradation, and cuvette variation.

Slight errors or variation within the fluorescence detected in the samples of tissues with a very low sample weight, such as the right kidney and heart tissues, mentioned earlier, have the potential to become greatly amplified when the mass specific perfusion is calculated. A slight contamination of the sample with microspheres or fluor, or a slight variation in the fluorimeter calibration, could easily overestimate the number of microspheres in the sample which when translated into mass specific perfusion becomes large.

While all possible care was taken in sample processing the high mass specific flow values observed in the right kidney and heart samples in chapters three and four, and the sample size of some organs needs to be considered as a limitation of the resolution of the method.

2.6.2 Behaviour of microspheres in *H. iris*

The application of the fluorescent microsphere method of determining regional blood flow in *H. iris* requires that certain criteria be satisfied for the distribution of the microspheres to accurately reflect the partitioning of cardiac output, and these assumptions and their validity are addressed as follows:

Microspheres do not enter the animal:

The potential for abalone to occlude blood vessels has been reported

(Bourne, 1974), and the potential for the efferent ctenidial vessel into which the microspheres were injected, to be cannulated incorrectly or for the cannula to become blocked also exists. Care was taken to ensure correct cannula placement and this was checked prior to injection. The number of microspheres recovered from the animal gives the best indication of the success of an injection, a low recovery indicating an injection which did not introduce all of the microspheres into the circulatory system. Data from animals in which low recovery was observed were not used in the reported experiments.

Possible causes of microspheres not successfully entering the animal include air bubbles being injected along with the microspheres, incorrect cannula placement, the animal forcefully occluding the vessel, or the cannula obstructing normal blood flow.

The microspheres must be mixed into and carried by the circulation.

The microspheres were injected into the circulation at a steady rate into the right efferent ctenidial vessel which feeds directly into the heart, where it was assumed that the microspheres became well mixed. The diversity in the number of tissues which received microspheres and the spatial heterogeneity observed in earlier sampling did not suggest that any clustering or aggregation was occurring. Microscopy did not reveal any aggregation of microspheres, thereby also indicating good mixing (Figures 2.7-2.10).

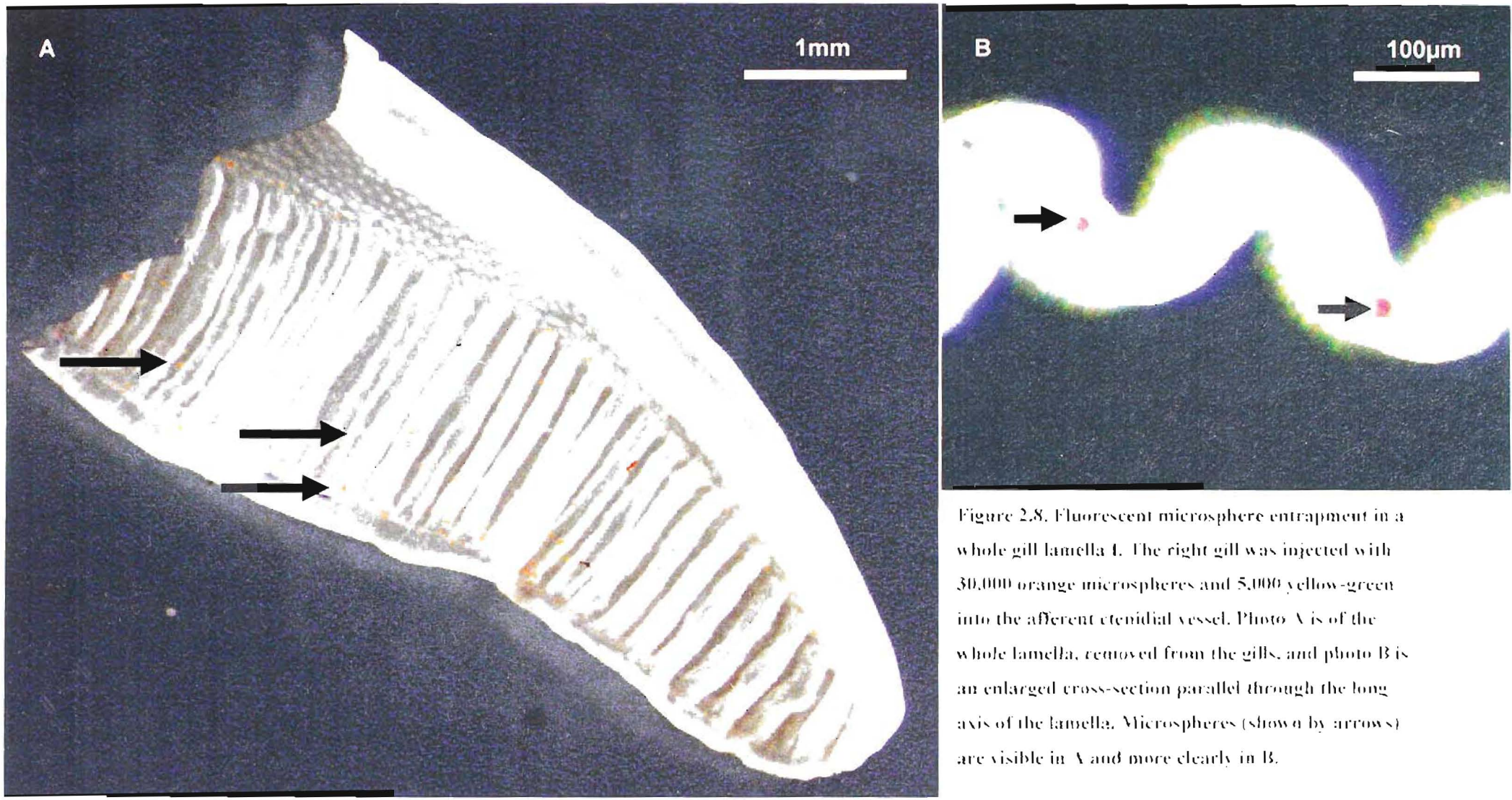


Figure 2.8. Fluorescent microsphere entrapment in a whole gill lamella 4. The right gill was injected with 30,000 orange microspheres and 5,000 yellow-green into the afferent etenidial vessel. Photo A is of the whole lamella, removed from the gills, and photo B is an enlarged cross-section parallel through the long axis of the lamella. Microspheres (shown by arrows) are visible in A and more clearly in B.

Figure 2.9. Fluorescent microsphere entrapment in a whole gill lamella II.

The view is of the ventral edge of the lamella in Figure 2.7.

The animal was injected with 30,000 of each colour (yellow-green and orange) into the afferent ctenidial vessel.

Tissue was fixed in Bouin's fixative and cleared using ethanol saturated with Li_2CO_3.

The arrows indicate the location of the some of the microspheres.

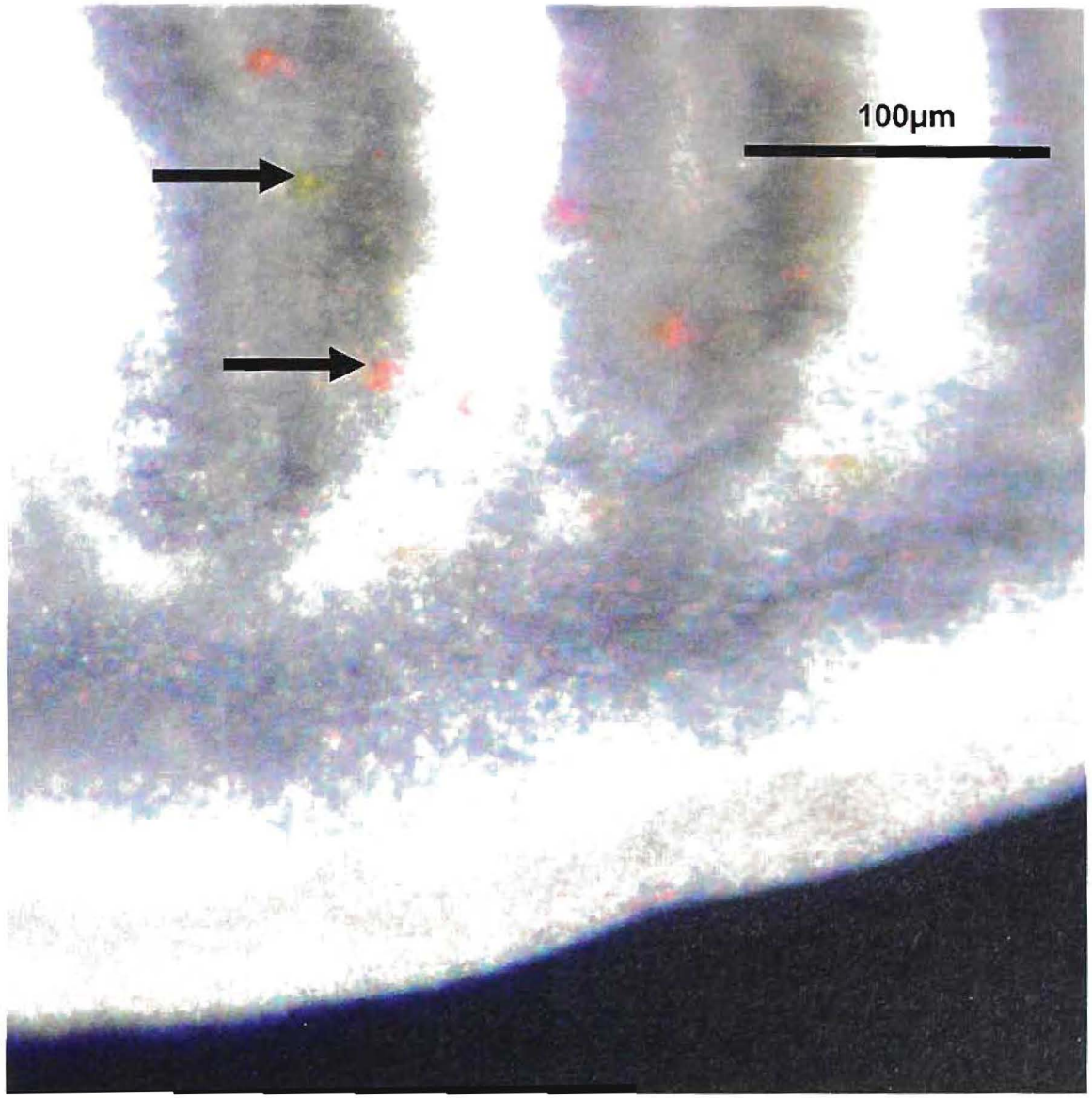


Figure 2.10. Fluorescent microsphere entrapment in a cross-sectioned gill lamella I.

The animal was injected with 30,000 of each colour (yellow-green and orange) into the afferent ctenidial vessel.

The arrows indicate the location of the some of the microspheres, which appear to be trapped within the narrow vasculature of the lamella.

Tissue was fixed in Bouin's fixative and cleared using ethanol saturated with Li_2CO_3. Sample staining is with Haematoxylin and Eosin. The dehydration and embedding process was done using Histoclear® II (xylene dissolves microspheres) and sections were mounted with a glycerine jelly temporary mount.

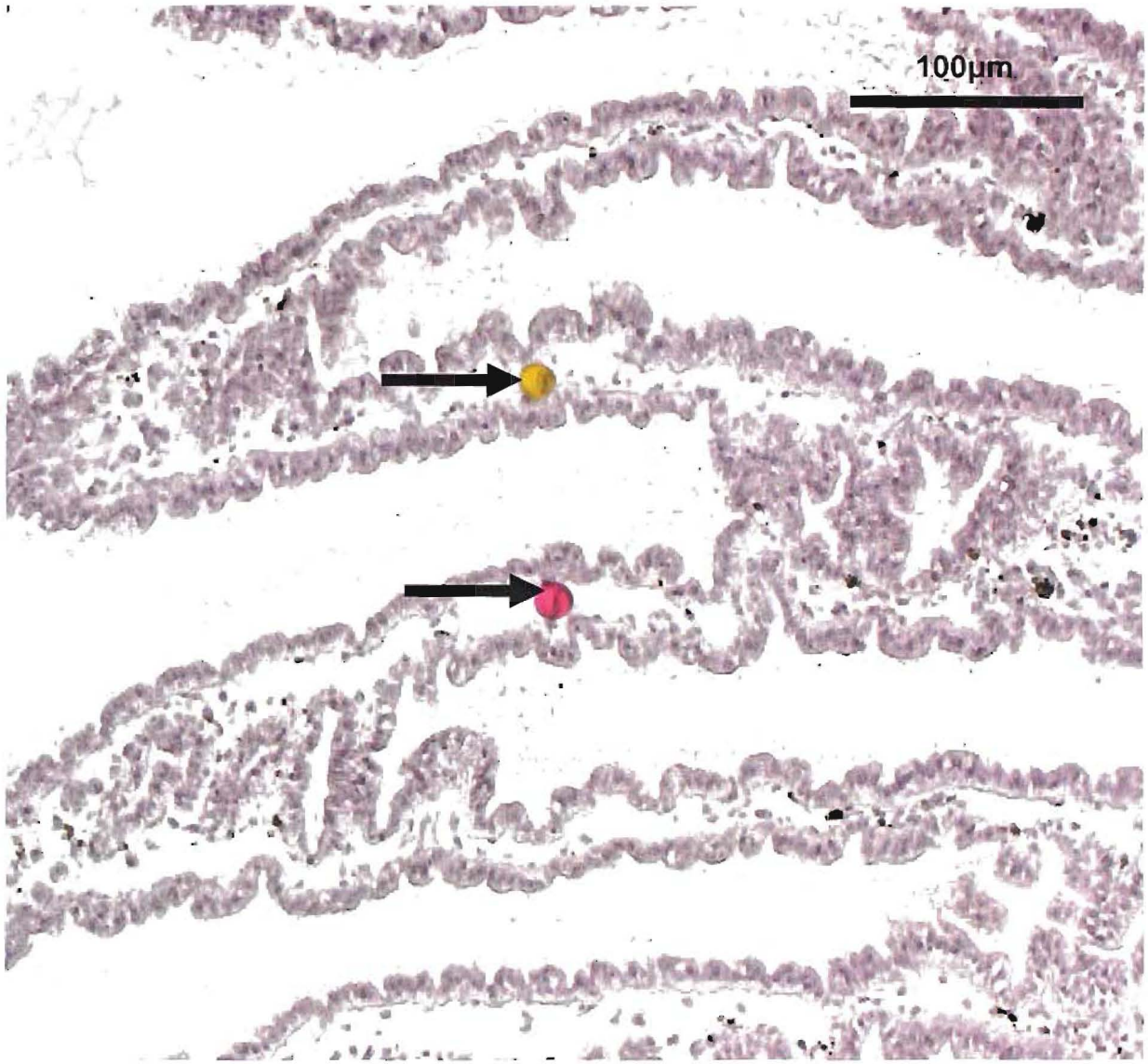


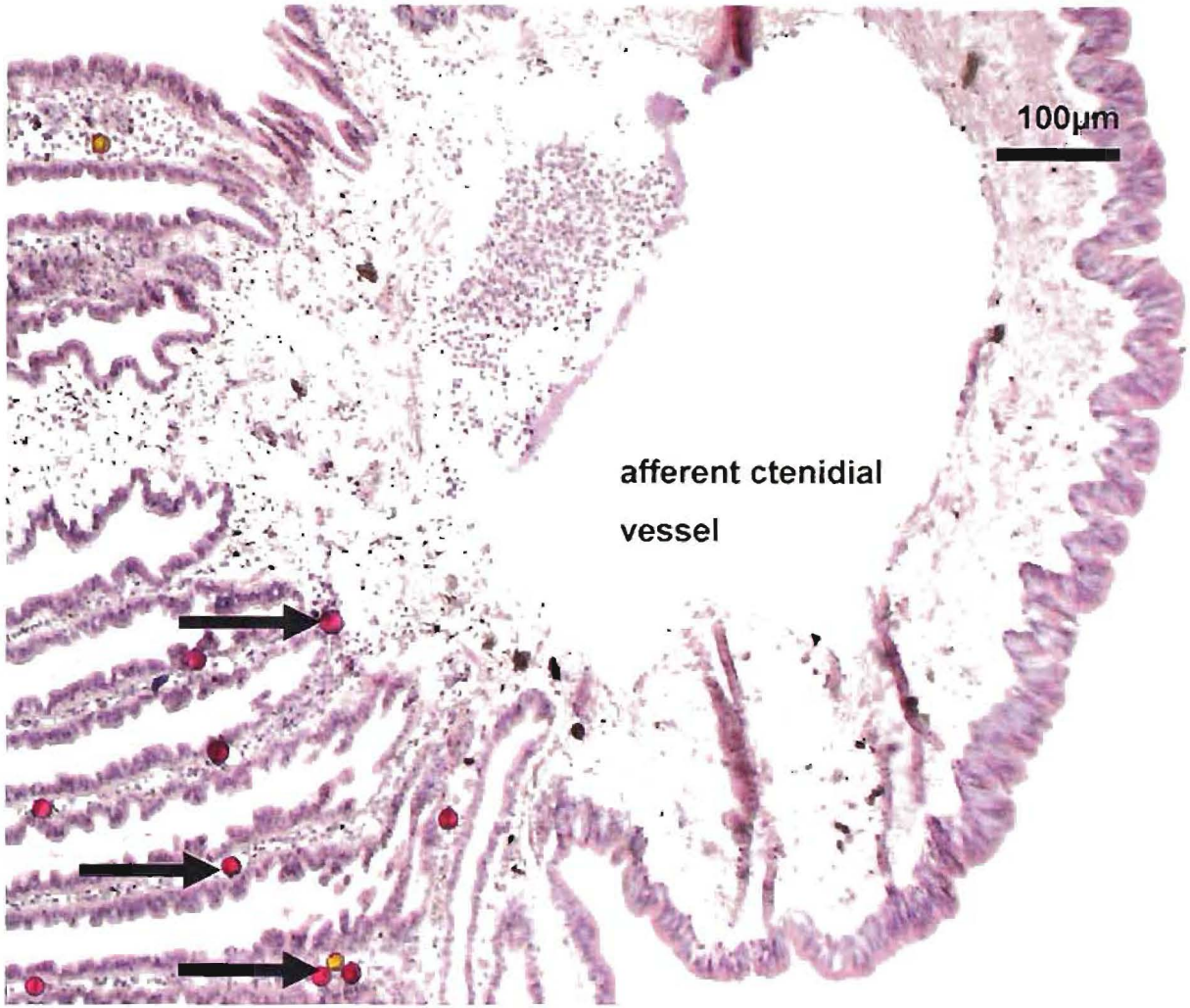
Figure 2.11. Fluorescent microsphere entrapment in a cross-sectioned gill lamella II.

The arrows indicate the location of the some of the microspheres.

The animal was injected with 30,000 of each colour (yellow-green and orange) into the afferent ctenidial vessel (labelled).

The arrows indicate the location of the some of the microspheres, which appear to be trapped within the narrow vasculature of the lamella.

Slides preparation was as described for Figure 2.9.



The microspheres become entrapped on the first pass.

The appearance of microspheres in the "venous" system of *H. iris* from an "arterial" injection was initially cause for concern. In mammalian studies, arterially injected microspheres are trapped in the capillaries of the arterial system and do not appear in the venous system, and significant venous entrapment indicates that the microspheres being used are too small. The appearance of microspheres to the venous system in *H. iris* is probably as a consequence of arterio-venous shunting, the implications of which are discussed further in chapters three and four, not because they are passing through the tissues without becoming entrapped.

It cannot be said for certain that the microspheres were trapped on the first pass through the circulatory system, but if microspheres which were not entrapped made their way back to the heart, then their pattern of redistribution would still be proportional to the relative perfusion of the respective tissues.

Microspheres entrapment is permanent and they are retained by all tissues

In vertebrates it is reported that microsphere entrapment is stable over days (Glenny et al., 1997), however this does not necessarily apply to abalone tissues.

The microsphere entrapment appears to be permanent and stable, as the observed microsphere distributions in later experiments (discussed in chapters three and four) were similar in control animals over a period of 10 minutes (Chapter four), and also over 24 hours (Chapter three). If microsphere entrapment was not permanent, and thus able change if the relative perfusion of a tissue changed, then the observed distribution of microspheres already entrapped in animals at the time a treatment was applied (emersion or clamping), would likely be different between control (resting) and the treatment animals. As this was not the case the microsphere entrapment appears to be permanent, and histological sections of the gills also support this, as the microspheres appear lodged in the vasculature (Figures 2.7-2.10).

Microspheres become entrapped and do not simply settle out

The microspheres could potentially settle out of the circulation, as opposed to becoming entrapped, in tissues or situations where low blood flow occurs. The likelihood of this occurring is low, as considerable surges of pressure recorded in equal magnitude from the arterial and venous systems of abalone during sudden movement such as muscular contraction (Bourne and Redmond, 1977a) would most likely remobilise microspheres which were merely settled, and as mentioned earlier the distributions of trapped microspheres appear unaffected by disturbing the animal.

Microspheres do not significantly increase peripheral resistance.

The possibility of microspheres lodging in the tissues causes alteration of localized haemodynamics or in extreme cases ischemia is remote. In an experiment by Deveci and Eggington (1999) rats of a slightly larger body weight (300-400g) to the abalone in the present study were injected with double the number of fluorescent microspheres without measurable alteration to the blood pressure or regional blood flow (Deveci and Eggington, 1999), and Glenny et al (2000) report minimal increases in peripheral resistance following sequential microsphere injections. This does not necessarily mean the same rules apply to the circulatory system of *H. iris*, which is completely different to that of vertebrates. The distribution of the microspheres reported in chapters three and four, are at extremely high levels in any one organ and probably do not cause a measurable increase in peripheral resistance.

As microsphere entrapment is relative to the perfusion of any given tissue, any consequent increase in overall peripheral resistance would be unlikely to alter the relative blood distribution to the tissues. Further experiments are required to ascertain if changes in haemodynamics occur as a result of microsphere injection.

The relative perfusion of the tissues reflects cardiac output.

As the microspheres are distributed to the tissues in the blood, the proportion of microsphere entrapment in any tissue should reflect the proportion of the cardiac output that tissue receives. This assumption depends on all tissues that receive blood being capable of trapping microspheres, and the distribution of the microspheres in the experiments reported in chapters three and four suggest that all tissues are capable of microsphere entrapment.

Whether some tissues facilitate entrapment more readily than others was not established and this assumption is a potential limitation of the experiments. The appearance of relatively large proportions of the microspheres in the venous tissues, such as the gills and right kidney (Chapters three and four), was initially thought to be leakage through tissues which did not trap them. However due to the stability of the distribution observed under different physiological states (Chapters three and four) the more likely scenario is that these "venous" tissues are actually receiving at least a proportion of the "arterial" blood supply. This possibility is discussed further in chapters three and four.

CHAPTER THREE

CIRCULATORY CHANGES DURING EMERSION

3.1 INTRODUCTION

Haliotis iris typically lives in the subtidal region of rocky coastlines but during extreme low tides individuals, juveniles particularly, are occasionally exposed to air (Poore, 1972b). Other prosobranch gastropods, such as the mesogastropod and neogastropod snails, are able to seal themselves entirely in a protective environment by retreating into the shell which is protected by an epiphragm when deprived of water and exposed to air and higher temperatures. Limpets (archaegastropoda), e.g. *Acmea. sp.*, are adapted to cope several hours of emersion on dry rocks by forming a relatively tight seal against the rock to prevent water loss, supplementing respiratory requirements by using the mantle, as the ctenidia do not function properly emersed (Kingston, 1968).

H. iris has a row of shell holes (tremata) running dorsal to the gills and is less capable than other gastropods of sealing itself off from the external environment. The predominantly subtidal habitat of *H. iris* (Poore, 1972a; Schiel and Breen, 1991) means it not likely to suffer long term exposure to air.

The demand for live abalone and increases in live-capture for brood and pearling stock has resulted in an increased need to live-freight specimens of *H. iris*, a process in which the animal may be without water for several hours (Baldwin et al., 1992; Wells and Baldwin, 1995).

When *H. iris* is submerged, the gills are suspended freely in the mantle cavity. A constant respiratory current, generated in part by cilia, enters the mantle cavity near the head, flows across the gills and exits via the tremata. When emersed, the animal is presumably incapable of generating this respiratory current. The emersed gills appear collapsed, less extended, and their effective surface area is greatly reduced. Emersed animals become hypoxic, presumably as a consequence of substantially reduced capacity for gas exchange (Behrens et al., 2002).

During live capture for experiments, specimens regularly experienced up to 12 hours of emersion and usually recovered without obvious problems, and prolonged emersion of 24 hours would likely occur in "dry shipping" of abalone overseas.

In the experiments reported in this chapter, changes in the relative perfusion of the peripheral tissues which could facilitate gas exchange, notably those of the foot, mantle and epipodium, were addressed by examining the partitioning of fluorescent microspheres before and after prolonged emersion in a double injection protocol.

Preliminary observations showed animals emersed for long periods of time protruded their epipodial and foot surfaces, and increased the exposure of the mantle to air, and thus it was hypothesised that the foot, mantle and epipodial surfaces contribute to gas exchange in an emersed hypoxic animal and that a redistribution blood flow associated with facilitation of gas exchange would result in an observation of increased entrapment of microspheres from the treatment injection in these tissues.

3.2 MATERIALS AND METHODS

3.2.1 Outline of treatment

The animals were set up with injection cannulae and an optical heart monitoring system to measure heart rate.

The first injection of yellow-green microspheres was under "control" conditions, with the animal in a relaxed state. The animal was left for 10 minutes for complete microsphere entrapment and then the water was drained from the containers, leaving the animals emersed. A second injection of orange microspheres was administered 24 hours later and the animals were left emersed for a further 10 minutes, at which time they were sacrificed.

The microsphere distribution and cardiac output measurements from the first injection of yellow-green microspheres are expected to reflect the state of the circulation in a resting animal, and correspondingly, the second injection of orange microspheres should reflect the state of the circulation in an animal under prolonged emersion.

The following methods outline the preparation and treatment of the animal for the emersion experiment. For more detailed aspects of animal preparation and experimental setup the reader is directed to chapter two.

3.2.2 Animal preparation

Animals of a healthy appearance ranging from about 120mm to 170mm in shell length and 222g to 513g total weight, were selected at random from their holding tanks. 10 animals were used in the 24hr emersion protocol and 4 "control" animals were injected with microspheres at the same times but remained immersed for the whole 24hrs.

The cannulation procedure was described in chapter two. To summarise, the first cannula was inserted retrograde into the aorta approximately 20mm anterior to the heart, and the second inserted orthograde into the right efferent ctenidial vessel approximately 30mm anterior to the heart. Both cannulae were fed in approximately 5-10mm to ensure that they remained in the vessel if the sites of insertion moved relative to the shell to which they were anchored.

Animals were each placed in a 2 litre plastic container, supplied with aerated seawater at 15°C from a re-circulating system, and left to rest for a minimum of 24 hours to allow recovery.

3.2.3 Microsphere injection

Prior to injection, cannula patency was checked by withdrawing about 20µl of haemolymph from the efferent ctenidial cannula, and 100µl from the aortic cannula, using a 100µl Hamilton gastight syringe. The aortic cannula sample was set aside to flush the cannula after the microsphere injection.

The injectate was prepared as described in chapter two, in Instant Ocean™ (Aquarium systems) saline isosmotic to seawater and 0.1% Tween80® at a density of 400 microspheres per µl. An injection volume of 250µl (100,000 microspheres) was drawn up into a 250µl SGE glass syringe and the needle inserted into the right efferent ctenidial cannula. The syringe was placed in a vertically mounted syringe pump (Sage Instruments) and the microsphere solution (250µl) was injected into the animal over 1.5min.

After the microspheres were injected, the 100µl of blood withdrawn earlier was injected using the syringe pump, to flush any remaining microspheres through the cannula.

The empty 250µl syringe was filled twice with distilled water and each time discharged quickly into a sample tube to establish how many microspheres which were not injected into the animal remained in the syringe.

3.2.4 Emersion and second injection

After the first injection, the animals were left for 10 minutes and then the water from their containers was drained and they remained emersed for 24 hours. A second injection of orange microspheres was administered using the same procedure and preparation as the first injection. The emersed animals were in very moist conditions at all times and kept at 12°C.

3.2.5 Sacrifice

After the second injection the animals were left for 10 minutes to allow complete microsphere entrapment. They were then placed into a large volume (5L) of 50°C seawater, in which they died quickly. The dead animals were placed whole in individual "zip-lock" plastic bags and stored at 4°C until they were ready for dissection. The justification for using this method of sacrificing the animals is discussed in chapter two.

3.2.6 Tissue sampling

The total tissue weight of the animal was obtained by subtracting the shell weight from the total weight, and ranged from 139g to 353g.

After separation by dissection, small organs were placed whole into pre-weighed 14ml polypropylene screw-cap tubes. Larger tissue and organs that were too large to be sampled in their entirety were weighed, homogenised with a known volume of distilled water, and a sub-sample of the homogenate was placed into the sample tubes. The organs sampled whole were the heart, left kidney, left ctenidia, mucous gland, right ctenidia, and as entirely as was possible, the right kidney. The organs which required homogenisation and sub-sampling were the adductor muscle, digestive gland and gonad, epipodium, foot muscle, head, mantle. The

remainder of non-characterisable tissue, consisting mostly of intestine and visceral tissue, was labelled "intestine and other viscera".

Tissue sampling and homogenisation is discussed in more detail in chapter two.

3.2.7 Microsphere extraction and fluorimetry

Samples were digested in 2M ethanolic KOH and microspheres extracted in 3ml of Cellosolve® acetate using the sedimentation method as described in chapter two.

Sample fluorescence was measured in 10mm path fluorometric glass cuvettes by a Varian (Cary Eclipse) fluorescence spectrophotometer. The fluorescence intensity in arbitrary units (a.u.) of a sample was proportional to the number of fluorescent microspheres dissolved in it, and using standards of known microsphere concentrations, the number of microspheres in each sample, and thus in each tissue or organ, was determined.

The calculations of the microsphere density in a tissue based on the sample fluorescence are shown in chapter two.

3.2.8 Measurement of regional organ perfusion

Differential perfusion is expressed as the number of microspheres per organ as a percentage of the total number of microspheres recovered from the whole animal, or as mass specific blood flow (percent of recovered microspheres per gram tissue). The percentages of the total recovered microspheres are assumed to represent percentages of cardiac output (see chapter two).

3.3 RESULTS

3.3.1 Effect of emersion

The control (immersed) animals appeared healthy after the second injection and had a normal heart rate of approximately 23 beats per minute and the epipodial tentacles were protruded (Figure 3.1 A). In animals that had been without water for 24 hours, the foot and epipodium often protruded beyond the edge of the shell, and the epipodium appeared swollen (Figure

3.1B). The animals seemed to have lost muscular tone in the foot, as the shell was leaning toward the posterior right hand side, exposing the mantle. The heart, gills and other viscera had retracted or collapsed away from the shell wall, and the gills appeared collapsed and shrunken in the absence of support from the aqueous medium. The heart was still visibly beating, albeit at a noticeably slower rate than the controls, but the optical probe was unable to detect a heart rate due to the retraction of the heart from the shell surface.

All the control animals survived the 24 hours, and despite a somewhat flaccid appearance, none of the 24 hour emersed animals died.

3.3.2 Microsphere recovery

Microsphere recovery from the animals was assessed as the sum of all the microspheres in the sampled organs of an animal (Chapter two). Injections with low estimated recovery were removed from the reported means and 9 of the 10 yellow-green, and 7 of the 10 orange injections had an acceptable recovery (>50% label recovery). The average microsphere recovery in the included series was 83±7.6% yellow-green and 76±11.4% orange.

3.3.3 Stability of the microsphere distribution over a 24 hour delay

In the control treatment, the yellow-green and orange microspheres had a similar distribution (Figure 3.2) and mass specific flow (Figure 3.3) in most tissues even though they represented injections that were 24 hours apart. This supports two critical assumptions; firstly that the distribution of the microspheres is stable over 24 hours, and secondly that entrapment occurs within 10 minutes of injection. If the first assumption regarding stability of the distribution over time were false then we would expect an accumulation of microspheres in some tissues and a concurrent dispersal of microspheres from others. If the second assumption were untrue, lower numbers of microspheres from the second injection (orange) would have been expected in many tissues.

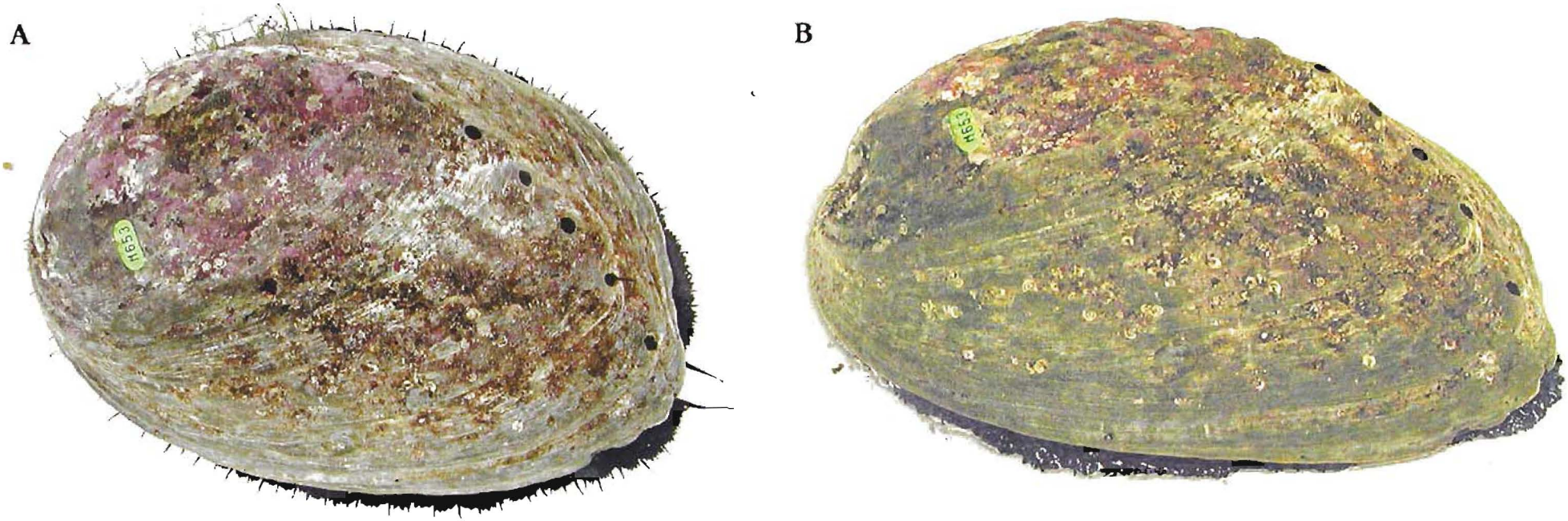


Figure 3.1. Photos showing a typical animal resting (A) and emersed (B). Note the protrusion of the epipodial tentacles and the two anterior cephalic tentacles in the resting animal (A). The animal protruded its foot and epipodium during emersion (B), and animals used in experiments were larger than that shown and this effect appeared more pronounced.

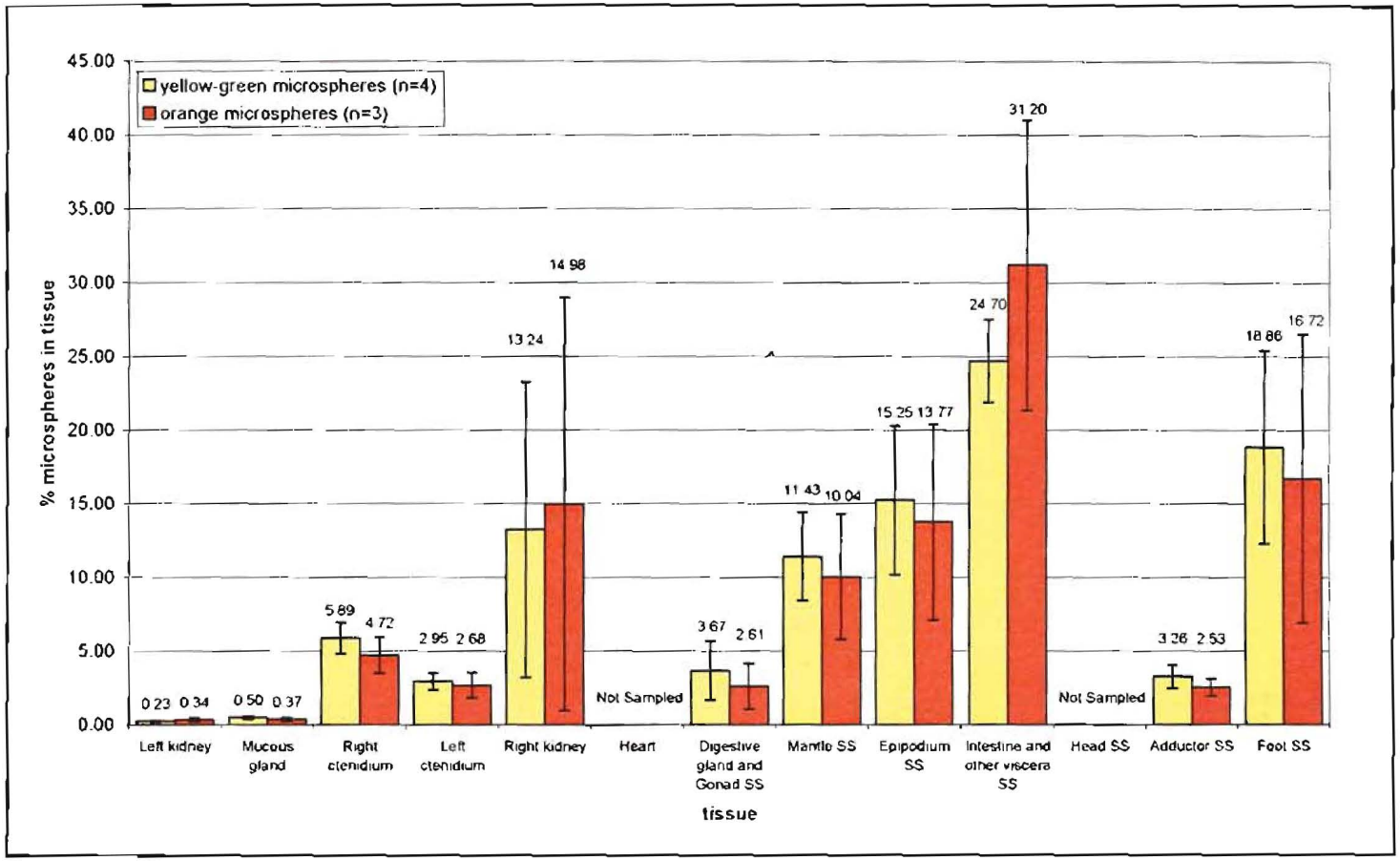


Figure 3.2. Partitioning of cardiac output among selected organs in resting immersed animals as indicated by distribution of two colours of 15μm diameter fluorescent microspheres injected 24 hours apart. The first injection was of yellow-green microspheres and the second, 24 hours later, of orange microspheres. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovery for the respective injections and the error bars denote the standard error of the means. Homogenised and sub-sampled tissues (x-axis) are denoted with SS. The similarity between the distributions of the two different colours indicates their stability of entrapment over 24 hours.

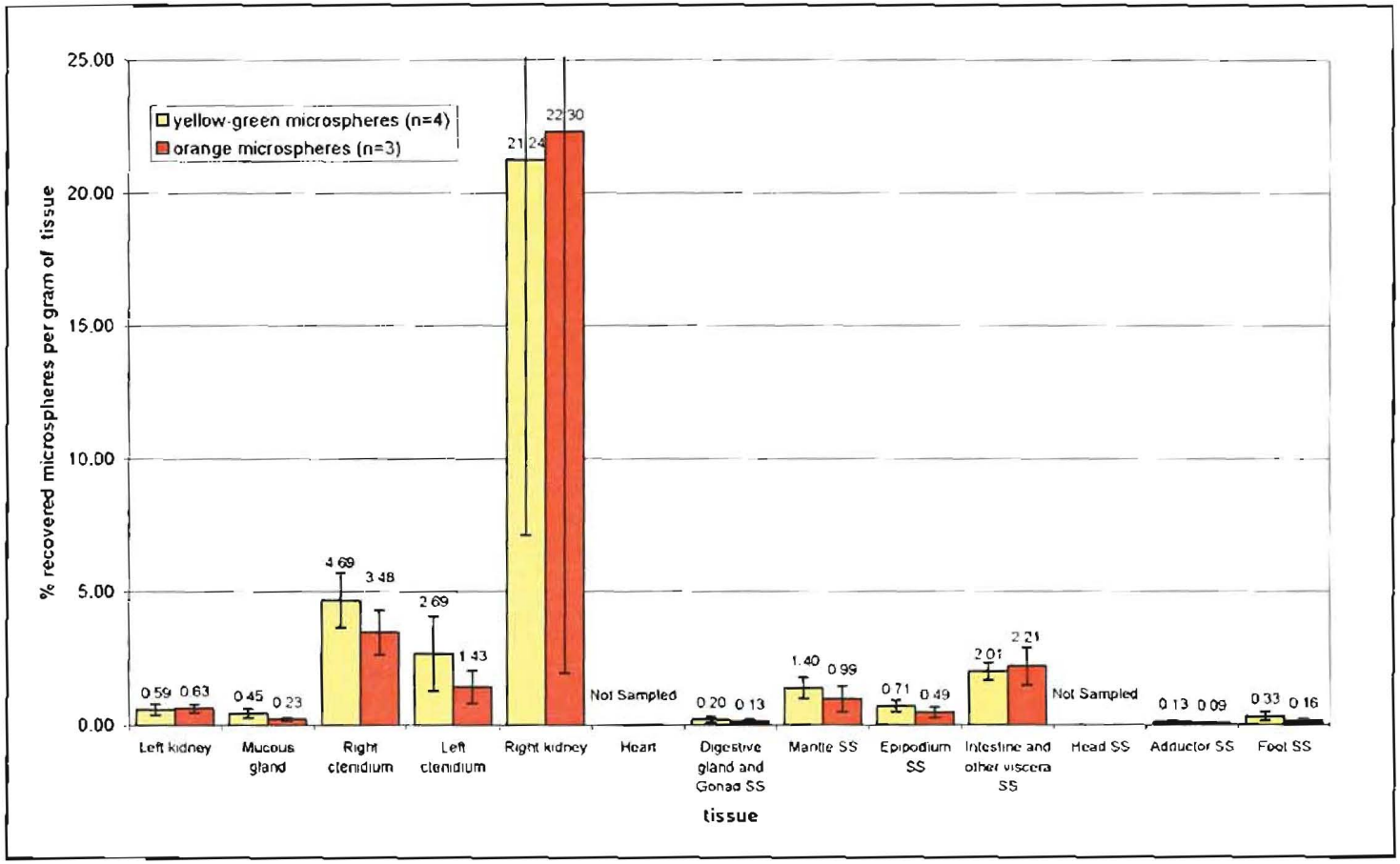


Figure 3.3. Mass specific distribution of cardiac output among selected organs in resting immersed animals as indicated by distribution of two colours of fluorescent microspheres injected 24 hours apart. The first injection was of yellow-green microspheres and the second, 24 hours later, of orange microspheres. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovery per gram tissue, for the respective injections and the error bars denote the standard error of the means. Homogenised and sub-sampled tissues (x-axis) are denoted with SS.

3.3.4 The partitioning of cardiac output in resting immersed paua

The highest mass tissue specific flow was in the right kidney (Figure 3.3; YG 21.24±14.1%, OR 22.30±20.4%), but two high values (63% yellow-green and 63% orange), still within the range of the later emersion values (Figures 3.4 and 3.5), contribute to a comparatively large proportion of this and account for the very high variability (Figure 3.3). This tissue also received a relatively large proportion of the total flow (Figure 3.2; YG 13±10.0%, OR 15 ±14.0%) with the same high variability (Figure 3.2).

Difficulties in dissecting the right kidney sample typically resulted in an incomplete and small sample and the potential errors which could result are discussed in chapter two.

The intestine and other viscera tissue received the highest proportion of the total recovered microspheres of both colours (Figure 3.2; YG 24.7 ±2.8%, OR 31.2±9.8 %) with higher variability in the orange recovery, and no difference was observed between the relatively high yellow-green and orange mass specific recoveries (Figure 3.3; YG 2.0±0.3%, OR 2.2±0.7%).

The left kidney and mucous gland samples each received few microspheres, both as a proportion of the total recovered (Figure 3.2; YG and OR less than 0.5%) and as mass specific flow (Figure 3.3; YG and OR less than 0.7%).

The right ctenidium samples weighed about two thirds of the left (right 1.4±0.04g, left 2.1±0.08g), but the relative and mass specific flows were about double (Figures 3.2 and 3.3). It is interesting that microsphere entrapment is observed in the gills, as this tissue is conventionally thought of as being venously supplied, and this suggests that the tissue actually receives arterial blood via an arterio-venous pathway, the implications of which are discussed later in this chapter.

The largest organ of *H. iris* is the foot, comprising 39±2.1% of the total tissue weight in the animals sampled in experiments. This tissue received less than 20% of the relative perfusion from both injections (Figure 3.2) and had a very low mass specific perfusion (Figure 3.3; YG 0.33±0.17%, OR 0.16±0.07%).

The adductor muscle had the lowest mass specific perfusion of any tissue sampled (Figure 3.3; YG 0.13±0.03%, OR 0.09±0.02%), and one of the lowest relative perfusions (Figure 3.2; less than 4%).

The digestive gland and gonad received few microspheres both in relative (Figure 3.2; less than 7%) and mass specific terms (Figure 3.3; less than 0.6%).

Some of the smaller organs of the animal, notably the epipodium (14±1.04% total weight) and more so the mantle (5.4 ±0.25% total weight), received relatively large relative perfusion (Figure 3.2; epipodium about 15%, mantle about 11%). Mass specific perfusion in the epipodium was relatively low at about half that of the mantle (Figure 3.3).

3.3.5 Changes in the partitioning of cardiac output in paua associated with 24hr emersion

Emersion changed the blood flow to specific tissues in *H. iris*. Increases in relative perfusion during emersion were observed in the mucous gland, both ctenidia, left kidney and heart, while concurrent decreases in right kidney and foot muscle were observed. Several tissues, adductor muscle, digestive gland and gonad, and interestingly the mantle and epipodium, showed no changes in relative perfusion during the emersion treatment.

The intestine and other viscera sample had the highest overall entrapment and a similar proportion of each injection (resting and emersion) was recovered, both in relative (Figure 3.4; YG 19.5±5.05%, OR 22.8±7.16%) and mass specific (Figure 3.5; about 2.5%) terms, with a high variability.

The higher mean flow to the mucous gland in emersed animals (Figure 3.4; OR 1.92±1.08% c.f. YG 0.9±0.19%) resulted from a single high value of 7.9% from the orange recoveries. The mass specific emersion treatment values are similar to the actual entrapment values and no difference is clear (Figure 3.5)

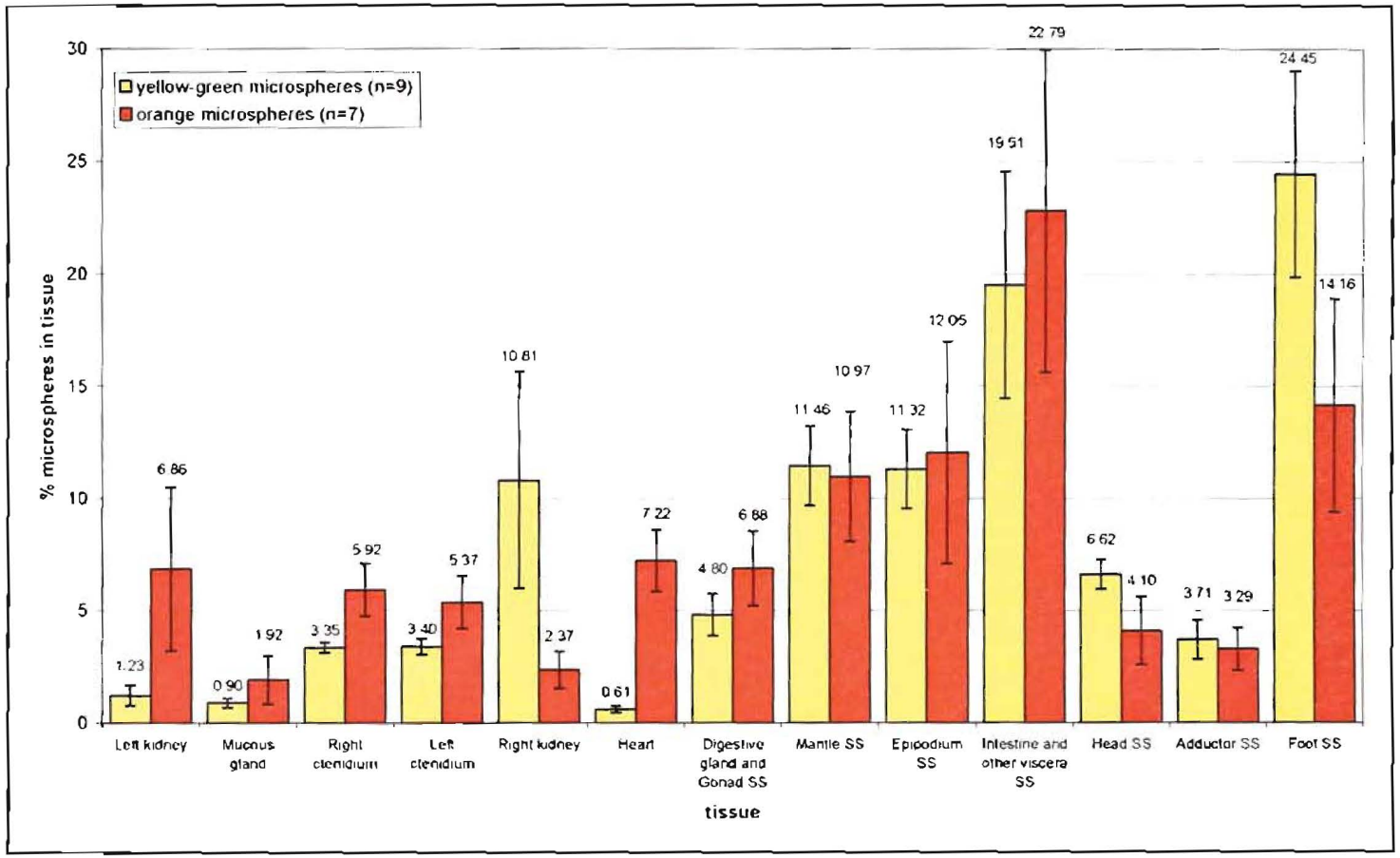


Figure 3.4. Changes in the partitioning of cardiac output among selected organs in emersed animals as indicated by distribution of two colours of fluorescent microspheres injected 24 hours apart. The first injection of yellow-green microspheres was into resting immersed animals. The second injection of orange microspheres followed 24 hours of emersion. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovered from the whole animal for the respective injections and the error bars denote the standard error of the mean. Homogenised and sub-sampled tissues (x-axis) are denoted with SS. The differences between the distributions of the two colours of microspheres reflect changes in the relative perfusion of tissues between immersed and emersed states.

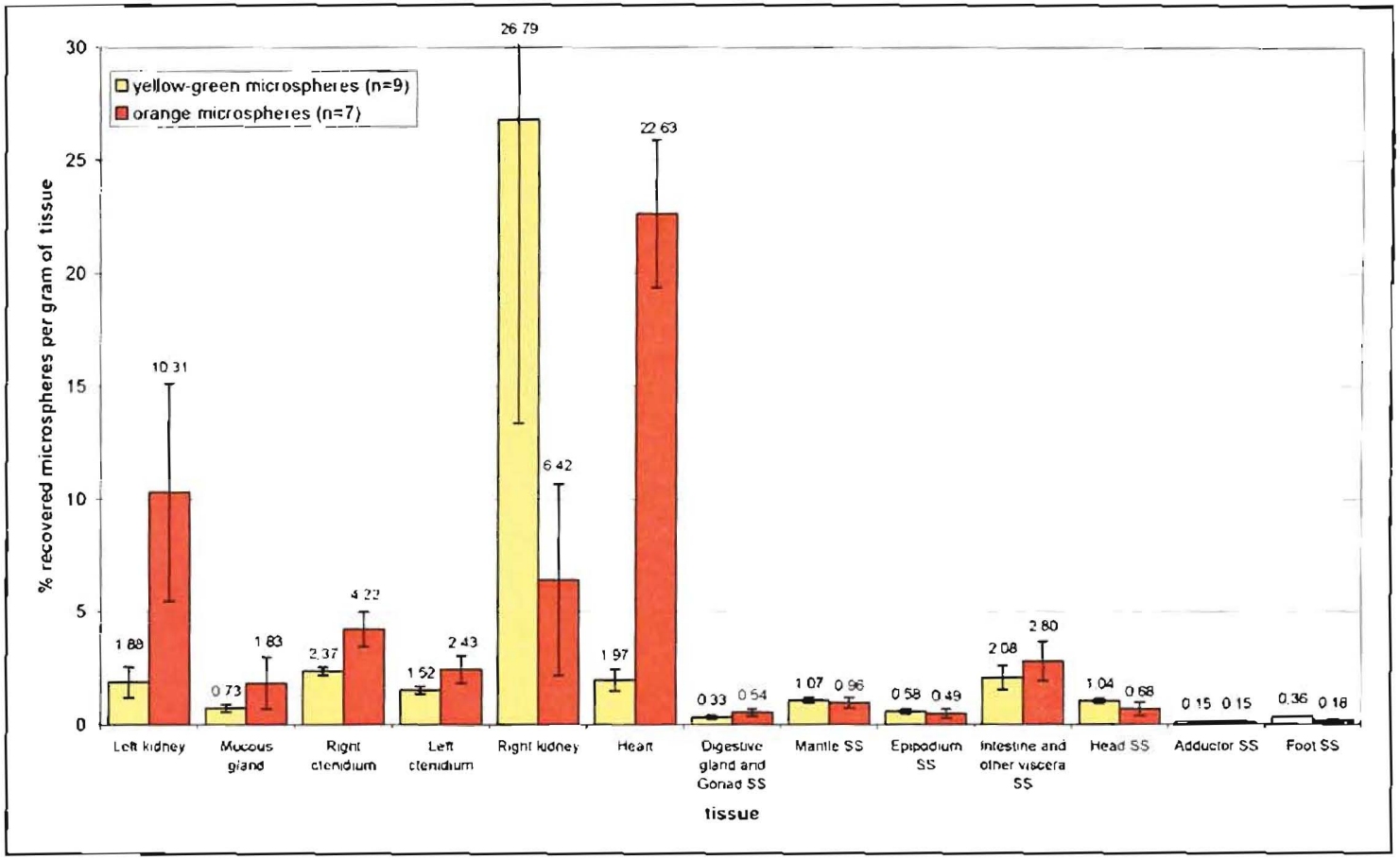


Figure 3.5. Changes in mass specific partitioning of cardiac output among selected organs in emersed animals as indicated by distribution of two colours of fluorescent microspheres injected 24 hours apart. The first injection of yellow-green microspheres was into resting immersed animals, the second injection of orange microspheres was following 24 hours of emersion. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovery per gram tissue for the respective injections, and the error bars denote the standard error of the means. Homogenised and sub-sampled tissues (x-axis) are denoted with SS. The differences between the distributions of the two colours of microspheres reflect changes in the mass specific perfusion of tissues between immersed and emersed states.

In the emersed state the relative blood flow to both the gills appeared to increase, with more microspheres entrapped during the treatment injection (Figures 3.4 and 3.5; OR c.f. YG). The right ctenidia received 3.4±0.23% of the yellow-green (resting) and 5.9±1.18% of the orange (emersed) injections, and the larger left ctenidia 3.4±0.35% of the yellow-green and 5.4±1.17% of the orange recovered microspheres (Figure 3.4).

A large increase in relative flow to the left kidney was observed (Figures 3.4 and 3.5) (YG 1.2±0.46%, OR 6.9±3.66%), but a single high orange entrapment value of 27.7% exaggerated the observed change, thus the large standard error (Figures 3.4 and 3.5).

The right kidney flow appeared higher but more variable in the emersed animal (Figures 3.4 and 3.5). The resting (yellow-green) entrapment ranged from 0.8% to 36.0% with a mean of 10.81±4.82%, and the emersed entrapment ranged from 0.75% to 6.12% with a mean of 2.4±0.82% (Figure 3.4).

The small sample weight of the right kidney and heart tissues resulted in very high mass specific flows observed in these tissues (Figure 3.5), and the potential for this to occur is discussed in chapter two.

A large increase in entrapment from the emersion injection was observed in the heart, which received a relatively few yellow-green microspheres (0.6±0.15%) but a large proportion of the second (orange) injection (7.22±1.37%) and mass specific flow (Figures 3.4 and 3.5). The greater observed microsphere entrapment in the heart tissue from the second injection was initially thought due to microspheres settling out shortly after injection due to a decreased blood throughput, however, as discussed in chapter two, because of an observed heart rate and the slow rate of injection this is unlikely. The heart rate was slower in the emersed animals (unpublished observation) but during the later reported clamping experiments, where a decrease in heart rate was measured, this appeared to have no effect on the microsphere mixing and distribution in the circulation.

Several tissues showed no change in relative perfusion during emersion.
The adductor muscle had a low relative perfusion (Figure 3.4; YG 3.71±0.87%, OR 3.29±0.95%) and a very low mass specific perfusion (Figure 3.5; YG 0.15±0.03%, OR 0.18 ±0.06%), both of which remained unchanged during emersion.

The digestive gland and gonad samples received similarly few microspheres from both injections (Figure 3.4; YG 4.8±0.93%, OR 6.9±1.67%) and a low mass specific perfusion (Figure 3.5; less than about 0.5%).

As mentioned earlier, it was hypothesised that in emersed animals the observed protrusion of the foot and epipodium, and increased exposure of the mantle surfaces would be associated with an increase of relative perfusion to these tissues to augment gas exchange.

Contrary to the hypothesis, no difference was observed between the relative blood flows from the first (immerse) and second (emersed) injections of microspheres in either the mantle and epipodium samples, and no difference is apparent in the mass specific data either (Figure 3.5).

The mantle received a similar relative perfusion (Figure 3.4) of 11.5±1.76% from the first injection (yellow-green) and 11.0±2.89% from the second (orange), and similar mass specific perfusions of 1.07±0.12% and 0.96±0.23% from the first and second injections respectively (Figure 3.5).

The epipodium received a similar relative perfusion (Figure 3.4) of 11.32±1.75% from the first injection and 12.05±4.95% of the orange microspheres recovered, and similar mass specific perfusions of 0.58±0.11% and 0.49±0.19% from the first and second injections respectively (Figure 3.5)

While the mass specific perfusions of the epipodium, and more notably so, the mantle were reasonably high compared with other tissues (Figure 3.5), they appeared unaffected by the emersion treatment.

In the foot tissues a decrease in the both the relative and mass specific perfusion during the emersed state was observed. The number of orange microspheres entrapped was almost half that of the number of yellow green (Figure 3.4; YG 0.36±0.06, OR 0.18±0.06). The mass specific data for the foot, which are described earlier as being low compared to other tissues, underwent a similar decrease (Figure 3.5; YG 0.36±0.06%, OR 0.18±0.06%).

The hypothesis of an increased perfusion in the event that this tissue was helping to supplement gas exchange is rejected on the basis that the opposite, a decrease in flow, is observed. Furthermore, the hypothesis that the mantle and epipodial tissues also function as auxiliary gas exchange surfaces during emersion is rejected on the basis that their relative and mass specific perfusions remain stable during both immersed and emersed conditions to which the animals were subjected.

3.4 DISCUSSION

It is reported that other gastropods, indeed prosobranchs use auxiliary gas exchange surfaces, such as the mantle and foot during periods of emersion or hypoxia (Brown, 1984; Kingston, 1968). In the experiments reported in this chapter, hypoxia was not assessed directly but Behrens et al (2002) report that specimens of *H. iris* emersed under similar conditions for 24 hours accumulate the anaerobic metabolites D-lactate and tauropine in the foot, adductor muscle and blood.

If *H. iris* was capable of utilising the foot, mantle or epipodial surfaces to facilitate gas exchange in the event of hypoxia because of a decreased functionality of the gills, we hypothesised that an increased relative perfusion in these tissues would be observed. Such changes were not observed, and a decrease occurred in the relative perfusion of the foot.

Interestingly both the left, and to a lesser degree, the right ctenidia show a marked increase in microsphere entrapment on emersion. Ctenidial entrapment of microspheres is not simply a representation of relative perfusion, as in theory almost all the venous blood flow in *H. iris* passes through them.

A possible cause of the increased appearance of microspheres in the gills is blood flow through the hypothesised CAS-CVS shunt, while blood flow to the muscles is occluded (Bourne and Redmond, 1977a; Russell and Evans, 1989). The decreased relative blood flow to the foot and adductor tissues observed in emersed animals, and the increased ctenidial entrapment, provide additional support for the existence of this shunt.

The function of this shunt may be to isolate tissues with high anaerobic capacity from the main circulation, effectively increasing the perfusion of the gills and remaining aerobic tissues.

Tissues which require constant blood flow may include the left kidney, mucous gland, intestine and other viscera, mantle, epipodium and the ctenidia themselves, as no decrease in the relative perfusion of these tissues was observed during emersion (Figure 3.4). However, it is unknown whether there was a change in the absolute blood flow to these organs, as cardiac output was not measured. The observation of moderate bradycardia indicates that this may be the case.

No increase in blood flow to the right kidney was observed (Figure 3.4). This is surprising given the increase in ctenidial flow. If the afferent (renal portal) flow to the right kidney received essentially the entire venous drainage in passage to the gills as proposed by Crofts (1929), and more blood bypasses the foot and adductor muscle via the CAS-CVS shunt (Russell and Evans, 1989), we might have expected an increase in microsphere entrapment in the right kidney during emersion.

Possibly, the right kidney is not always the singular drain for the entire venous system and is bypassed under certain circumstances.

In summary, total cardiac output probably decreases in hypoxic animals and the animal may possibly bypass certain organs capable of functioning under hypoxic conditions (adductor and foot) (Baldwin et al., 1992; Wells and Baldwin, 1995) to make less blood flow go further. There may be merit in isolating hypoxic organs from the main circulation as it may stem the accumulation of anaerobic metabolites, the presence of which do not appear to assist in oxygen uptake across gas exchange surfaces.

The schematic of the circulation in *Haliotis sp.* described earlier (Figure 1.1) shows the mantle effectively bypasses the entire "arterial" and "venous" systems, as it is fed blood directly from the ventricle and drains into the right efferent ctenidial vein. Crofts (1929) suggests that the blood is oxygenated in the mantle, thus it effectively augments gas exchange. The observation in the present experiment showing that relative blood flow does

not change to the mantle provides no support for this role of the mantle, at least during emersion.

Further measurements of haemodynamics in this poorly understood vascular route would clearly be beneficial.

The decrease in muscular tone observed in the foot, its capacity for anaerobic metabolism (Baldwin et al., 1992; Wells and Baldwin, 1995), and present data indicating reduced perfusion during emersion suggest that it is unlikely to function effectively as a gas exchange surface. The foot is not extensively vascularised (Crofts, 1929), further decreasing the likelihood that it can function as an auxiliary gas exchange organ. It must be noted that, as the foot was homogenised, sub-sampled, and treated as a whole organ, changes in flow to the periphery could not be excluded.

Despite appearing to have a greatly reduced capacity for gas exchange, the ctenidia appear to remain the primary surfaces of gas exchange under emersed conditions. *H. iris*, unlike other gastropods, does not appear capable of augmenting gas exchange with peripheral organs. This is facilitated by greatly decreasing blood flow in the adductor and foot tissues.

It would be interesting to investigate the cardiac output between immersed and emersed states, and the time required to clear anaerobic metabolites and restore the circulation in the foot and adductor muscle to normal.

A greater understanding of the physiology of *H. iris* under extended periods of emersion and its ability to recover would certainly be of significant commercial interest, given the demand for transport of live abalone both in pearling and broodstock industries, and countries to which delivery requires long freighting, of inevitably emersed animals, and could lead to decreased mortality or development of more economical transportation measures (Baldwin et al., 1992; Gordon and Cook, 2001; Wells and Baldwin, 1995)

CHAPTER FOUR

CIRCULATORY CHANGES DURING CLAMPING

4.1 INTRODUCTION

Clamping is the primary defence mechanism available to *Haliotis iris*. When threatened it uses its adductor muscle and foot to form a powerful suction thereby adhering firmly to the substrate. The suction is so powerful that the animal cannot be prised from a smooth substrate without the use of considerable force. There is strong evidence to suggest that during clamping a number of changes occur in the circulatory system, including acardia and isolation of the adductor and foot tissues from the main circulation (Bourne, 1974; Bourne and Redmond, 1977a; Russell and Evans, 1989).

A number of vascular routes have evidently been reported or hypothesised in some species of *Haliotis*, without clear morphological evidence and have proved elusive in other species. For example, a vascular connection from the cephalic arterial sinus (CAS) to the ventrally located cephalic venous sinus (CVS) has been proposed. This shunt is thought to provide a vascular bypass of the foot, and presumably adductor muscles, during clamping (Bourne and Redmond, 1977a; Russell and Evans, 1989). Vascular corrosion casts have failed to convincingly demonstrate the presence or absence of such a shunt (unpublished observation).

Systemic circulatory changes associated with clamping are poorly understood, both in terms of blood distribution and haemodynamics. Blood flow to the gills is similarly not well documented. The left ctenidium is approximately 30% larger than its right counterpart, but it is unclear which one receives the greater flow of blood, or whether the blood flow changes to the gills when the animal clamps. It has been suggested that impaired ventilation during clamping may cause internal hypoxia (Ainslie, 1980). Circulatory changes during hypoxia associated with emersion were reported in chapter three.

In the experiments reported in this chapter, changes in the relative perfusion of the adductor muscle and foot were addressed by examining the partitioning of fluorescent microspheres at

rest and during clamping in a double injection protocol Absolute blood flow to tissues and cardiac output in both resting and clamped animals were estimated using the reference withdrawal method outlined in chapter two

Estimates of cardiac output and cardiac stroke volume (CSV) are scarce in literature on abalone, and as far as I could ascertain only two previous studies, with quite differing results, have measured these physiological parameters. Bourne (1974) reported mass specific cardiac outputs of 42ml $kg^{-1}min^{-1}$ and CSV of around 1ml (15°C) in specimens of *H. corrugata* about 450grams wet weight, whereas Jorgensen et al (1984) reported mass specific cardiac output as being around 140ml $kg^{-1}min^{-1}$ and CSV of about 1.5ml (16°C) for specimens of *H. cracherodii* of about the same size (400g). There are no published estimates of cardiac output in *H. iris*

Clamping is associated with circulatory changes in abalone. Pressure surges following clamping, periods of acardia (Bourne and Redmond, 1977a), closure of valves in the CAS and CVS (Crofts, 1929), and shunting between the two sinuses (Russell and Evans, 1989) have all been reported

It was hypothesised that a decrease in cardiac output would be observed during clamping, and an increase in relative flow through the putative CAS-CVS shunt should result in increased trapping of MS in microvascular beds in the venous return routes, primarily in the right kidney and the ctenidial tissues.

4.2 MATERIALS AND METHODS

4.2.1 Outline of treatment

The animals were set up with injection and reference withdrawal cannulae, and an optical heart monitoring system to measure heart rate

The first injection of yellow-green microspheres was under "control" conditions, with the animal in a resting state, and after the injection the animal was left for 10 minutes to allow complete microsphere entrapment The animals were then induced to clamp and the second injection of orange microspheres was administered and the animals were induced to clamp for a further 10 minutes post-injection to allow complete microsphere entrapment, after which time they were sacrificed.

The microsphere distribution and cardiac output measurements from the first injection of yellow-green microspheres are expected to reflect the "normal" state of the circulation in the animals at rest, and correspondingly, the second injection of orange microspheres should reflect the state of the circulation in the animals during clamping.
The following methods outline the preparation and treatment of the animal for the clamping experiment. For more detailed aspects of animal preparation and experimental setup the reader is directed to chapter two.
14 animals were used in the clamping experiment and two controls were run for comparison.

4.2.2 Animal preparation

Animals of a healthy appearance ranging from about 120mm to 170mm in shell length, and 204g to 435g total weight, were selected at random from their holding tanks. Up to 10 animals were experimented on at any one time, staggered by 15 minutes to allow the injections to be administered sequentially. Each animal was cannulated as described in chapter two, with the first cannula inserted retrograde into the aorta approximately 2cm anterior of the heart, and the second orthograde into the right efferent ctenidial vessel approximately 30mm anterior of the heart. Both cannulae were fed in approximately 10mm to ensure that they stayed patent if the sites of insertion moved relative to the shell to which they were anchored.
Animals were left to recover for a minimum of 24 hours, each in a 2 litre plastic container supplied with aerated recirculating seawater and checked to ensure that they were not unhealthy immediately following cannulation, and again prior to experimentation. Injured animals with graze or puncture wounds or a moribund appearance were not used in the experiment.

4.2.3 First microsphere injection (yellow-green)

Prior to injection, cannula patency was checked by withdrawing about 20µl of haemolymph from the efferent ctenidial cannula, and 100µl from the aortic cannula, using a 100µl Hamilton gastight syringe. The aortic cannula sample was set aside to flush the cannula after the microsphere injection.

The injectate was prepared as described in chapter two, in saline Instant Ocean™) of a similar osmolality to seawater and 0.1% Tween80® at a density of 400 microspheres per µl. An injection volume of 250µl (100,000 microspheres) was drawn up into a 250µl SGE glass

syringe and the needle inserted into the right efferent ctenidial cannula. The syringe was placed in a vertically mounted infusion pump (Sage Instruments) and the microsphere solution was injected into the animal over 1.5min.

After microspheres injection the 100µl of blood withdrawn earlier was injected to flush the cannula.

The empty 250µl syringe was filled with distilled water and discharged quickly into a sample tube to establish the number of microspheres that remained in the syringe after injection.

4.2.4 Clamping and second microsphere injection (orange)

After the first injection the animals were left for 10 minutes, and then induced to vigorously clamp by gently attempting to pry them from the substrate, at which time the second injection of orange microspheres was administered using the same procedure and preparation as the first injection. The animals were induced to clamp for the duration of the second injection, the syringe flush and for a further 10 minutes after, to allow the microspheres to become entrapped. All animals responded by clamping for the duration of the treatment.
The control group was injected with orange microspheres as per the first (yellow-green) injection and the animals were left in a resting state and not induced to clamp.

4.2.5 Heart rate monitoring

An infra-red optical probe mounted on the animal reported heart activity. The signal from the probe was amplified and plotted on a chart recorder over the duration of the experiment. The procedure for mounting and testing the optical probe is described in detail in chapter two.

4.2.6 Reference withdrawal

Reference withdrawals from the aortic cannula were commenced simultaneously with each microsphere injection and continued for 5 minutes to allow the injected microspheres to pass the withdrawal point.
The reference sample was withdrawn from the animal as explained in chapter two, by placing the outlet of the aortic cannula below the level of the animal causing the blood to siphon into

a 14ml polypropylene screw-cap sample tube without vacuum assistance, although passive flow was only attainable when the pressure head was negative.
Reference withdrawals were taken successfully from 3 clamping treatment animals during both yellow-green and orange microsphere injections.
The reference withdrawal technique and calculations of cardiac output and CSV are discussed in detail in chapter two.

4.2.7 Sacrifice

After the second injection the animals were left for 10 minutes to allow complete microsphere entrapment. They were then placed into a large volume (5L) of 50°C seawater, in which they died quickly. The dead animals were placed whole in individual "zip-lock" plastic bags and stored at 4°C until they were ready for dissection. The justification for using this method of sacrificing the animals is discussed in chapter two.

4.2.8 Tissue sampling

The total tissue weight of the animal was obtained by subtracting the shell weight from the total weight, and ranged from 132g to 296g.
After separation by dissection, small organs were placed whole into pre-weighed 14ml polypropylene screw-cap tubes. Larger tissue and organs that were too large to be sampled in their entirety were weighed, homogenised with a known volume of distilled water, and a sub-sample of the homogenate was placed into the sample tubes. The organs sampled whole were the heart, left kidney, left ctenidia, mucous gland, right ctenidia, and as entirely as was possible, the right kidney. The organs which required homogenisation and sub-sampling were the adductor muscle, the digestive gland and gonad, the epipodium, the foot, the head, the mantle, and the remainder of non-characterisable tissue, consisting mostly of intestine and visceral tissue, was labelled intestine and other viscera. An improved technique of dissection reduced the quantity of tissue not specifically identified and grouped into the intestine and other viscera sample, compared with the emersion experiment samples (Chapter three).
Tissue sampling and homogenisation is discussed in more detail in chapter two.

4.2.9 Microsphere extraction and fluorimetry

Samples were digested in 2M ethanolic KOH and microspheres extracted in 3ml of Cellosolve® acetate using the sedimentation method as described in chapter two.

Sample fluorescence was measured in 10mm path fluorometric glass cuvettes by a Varian (Cary Eclipse) fluorescence spectrophotometer. The fluorescence intensity in arbitrary units (a.u.) of a sample was proportional to the number of fluorescent microspheres dissolved in it, and using standards of known microsphere concentrations, the number of microspheres in each sample, and thus in each tissue or organ, was determined. The calculations of the microsphere density in a tissue based on the sample fluorescence are shown in chapter two.

4.2.10 Measurement of regional organ perfusion

Differential perfusion is represented as the number of microspheres per organ as a percentage of the total number of microspheres recovered from the whole animal, or as mass specific blood flow (percent recovered microspheres per gram tissue). These percentages were assumed to represent percentage of cardiac output (Chapter two) and converted to absolute flows (ml min^{-1}) using the cardiac output estimates derived from the animals that provided reference withdrawal samples.

4.3 RESULTS

4.3.1 Effect of clamping

The control animals appeared healthy after the two injections with the epipodial tentacles extended. The heart rate remained constant during and after the injections, at around 23bpm.

Prior to clamping and during the first injection the treatment animals had extended epipodial tentacles and had a mean resting heartbeat of 21.8±1.58bpm. During the second injection when the animals were induced to clamp, the epipodial tentacles were retracted (Figure 4.1) and the average heart rate was reduced to 15.9±2.34bpm.
The animals did not appear weak or flaccid after the 10 minute clamping treatment.

A typical resting (control) animal and the same animal clamped are shown in figure 4.1.

4.3.2 Microsphere recovery

Microsphere recovery from the animals was assessed as the sum of all the microspheres in the sampled organs of an animal (as described in Chapter two). Of the fourteen animals injected, three yellow-green and one orange series with very low recoveries (average 13%) were eliminated from the means. The mean microsphere recoveries in the included series were 90±4.9% yellow-green and 89±5.9% orange.

4.3.3 Stability of the distribution of microspheres over 10 minutes

It has already been established that the microspheres become entrapped and do not change in distribution over a 24 hour period (Chapter three) and thus it seemed unlikely that changes in the distribution of microspheres would occur over the 10 minute delay between the yellow-green and orange injections in the clamping controls. Similarity between the yellow-green and orange microsphere distributions, both in terms of partitioning of flow (Figure 4.2) and mass specific perfusion (Figure 4.3), supports this expectation, albeit within limited number of observations (n=2).

4.3.4 The partitioning of cardiac output in the control animals

The distribution of the relative perfusion in resting animals has already been reported (Chapter three). Mass specific perfusion to the intestine and other viscera sample (Figure 4.2) was considerably lower than was observed in the emersion controls (Chapter three), probably due the improved dissection procedure.

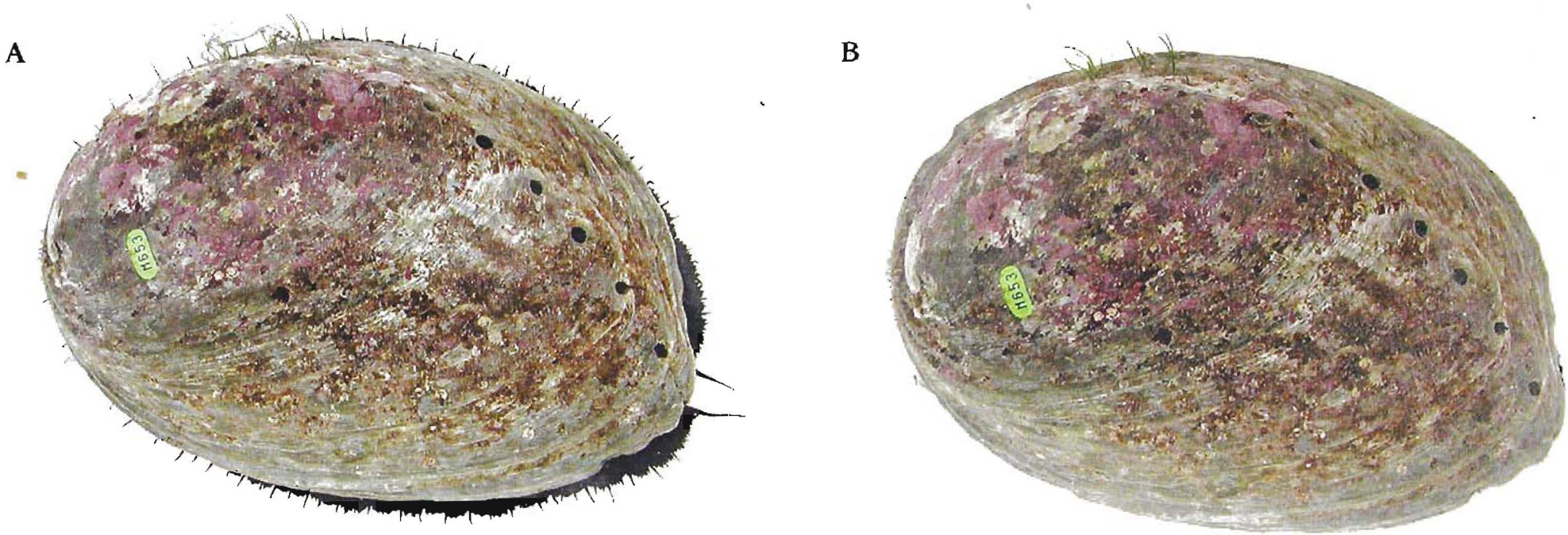


Figure 4.1. Photos showing a typical animal resting (A) and clamping (B). Note the protrusion of the epipodial tentacles and the two anterior cephalic tentacles which are retracted during clamping (B). Please note that this is the same animal which appeared in the photos reported in chapter three on the effect of emersion and photo A is the same as Figure 3.1. A (Chapter three).

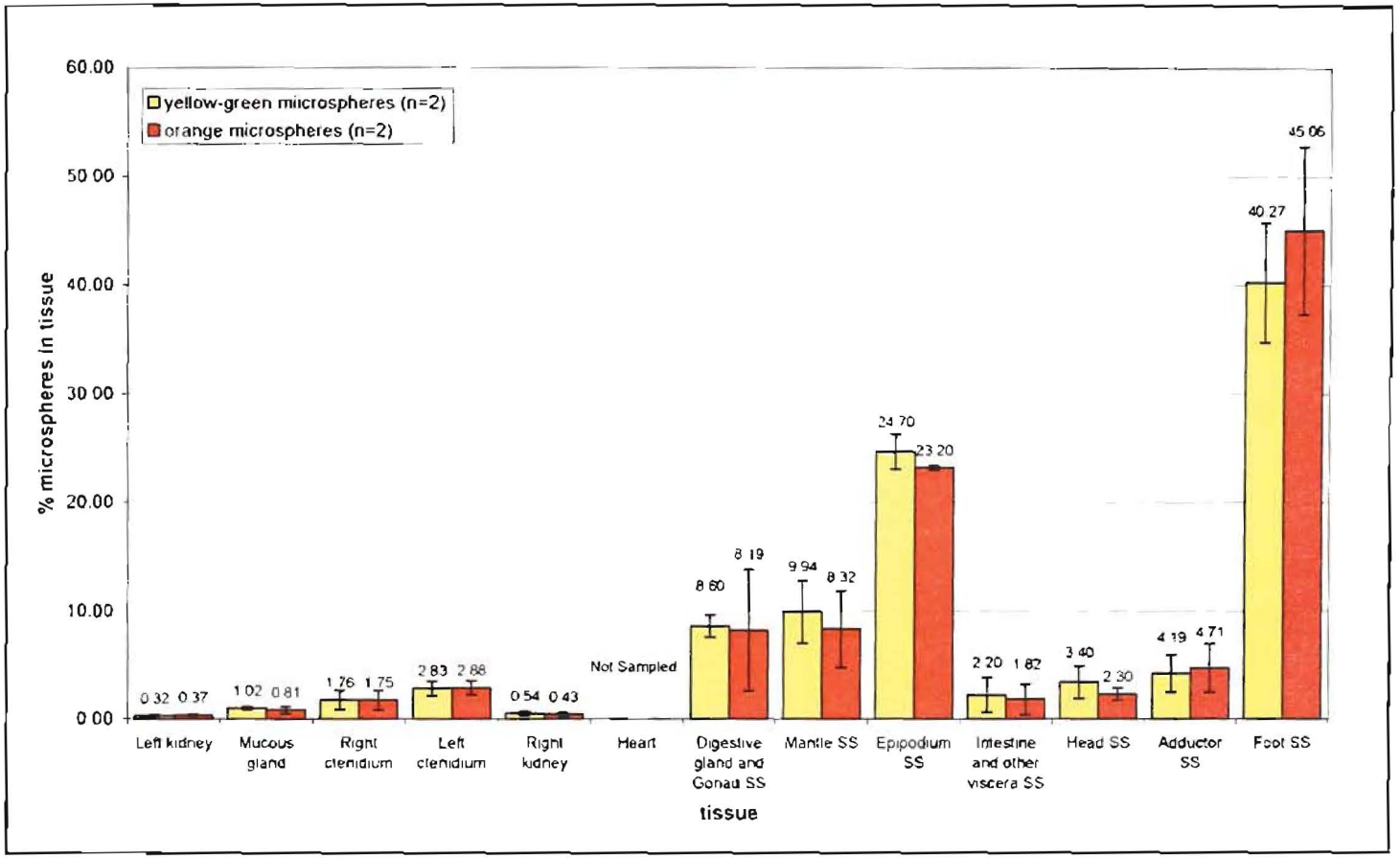


Figure 4.2. Partitioning of cardiac output among selected organs in resting animals as indicated by distribution of two colours of 15µm diameter fluorescent microspheres injected 10 minutes apart. The first injection was of yellow-green microspheres and the second, 10 minutes later, of orange microspheres. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovery for the respective injections and the error bars denote the standard error of the means. Similar distributions of yellow-green and orange indicate that the 10 minute delay did not result in movement of the microspheres from the first injection.

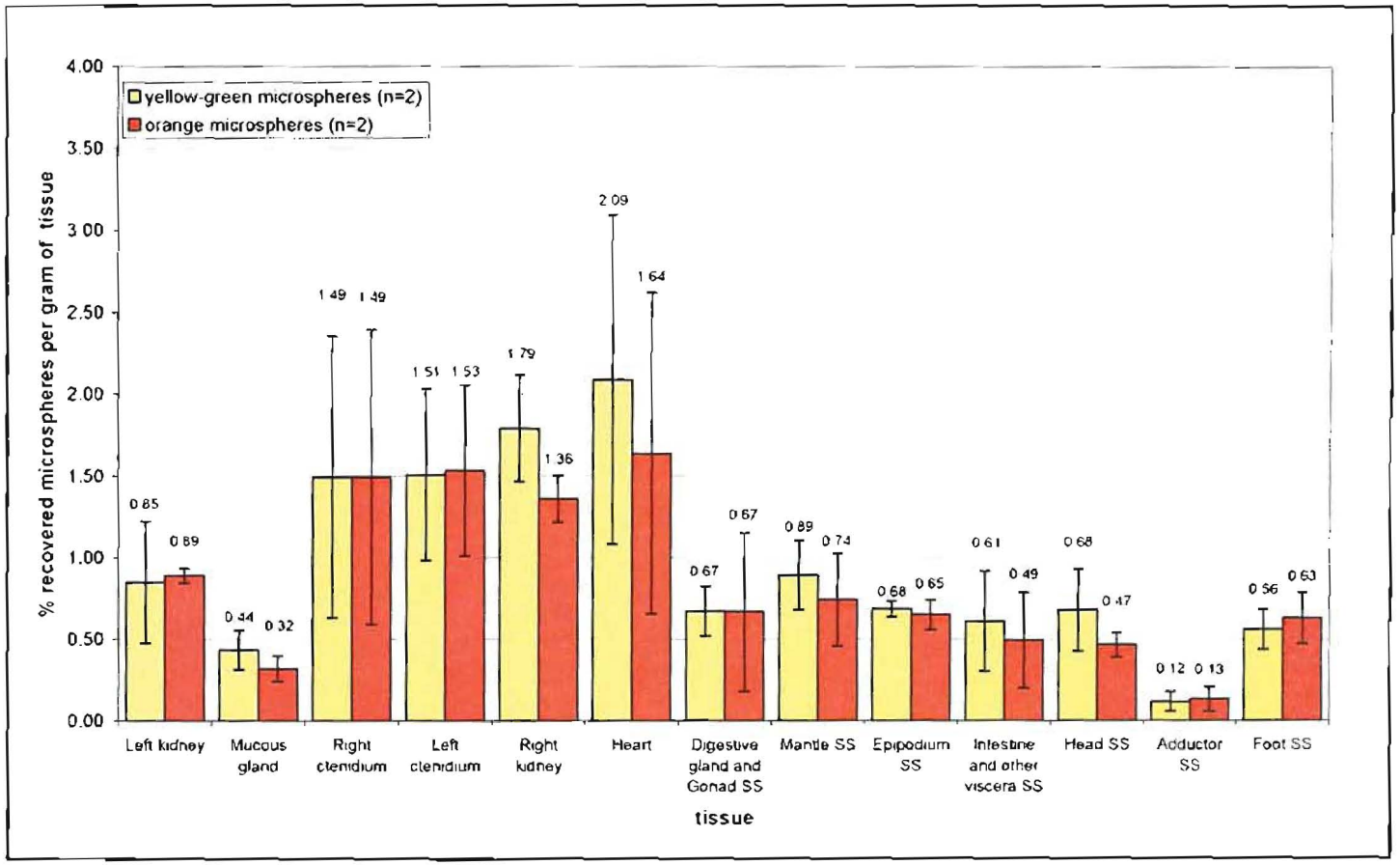


Figure 4.3. Mass specific distribution of cardiac output among selected organs in resting animals as indicated by distribution of two colours of fluorescent microspheres injected 10 minutes apart. The first injection was of yellow-green microspheres and the second, 10 minutes later, of orange microspheres. The animals were sacrificed 10 minutes after the second injection. The values are mean percentages of the total microsphere recovery per gram tissue for the respective injections and the error bars denote the standard error of the means. Similar distributions of yellow-green and orange indicate that the 10 minute delay did not result in movement of the microspheres from the first injection.

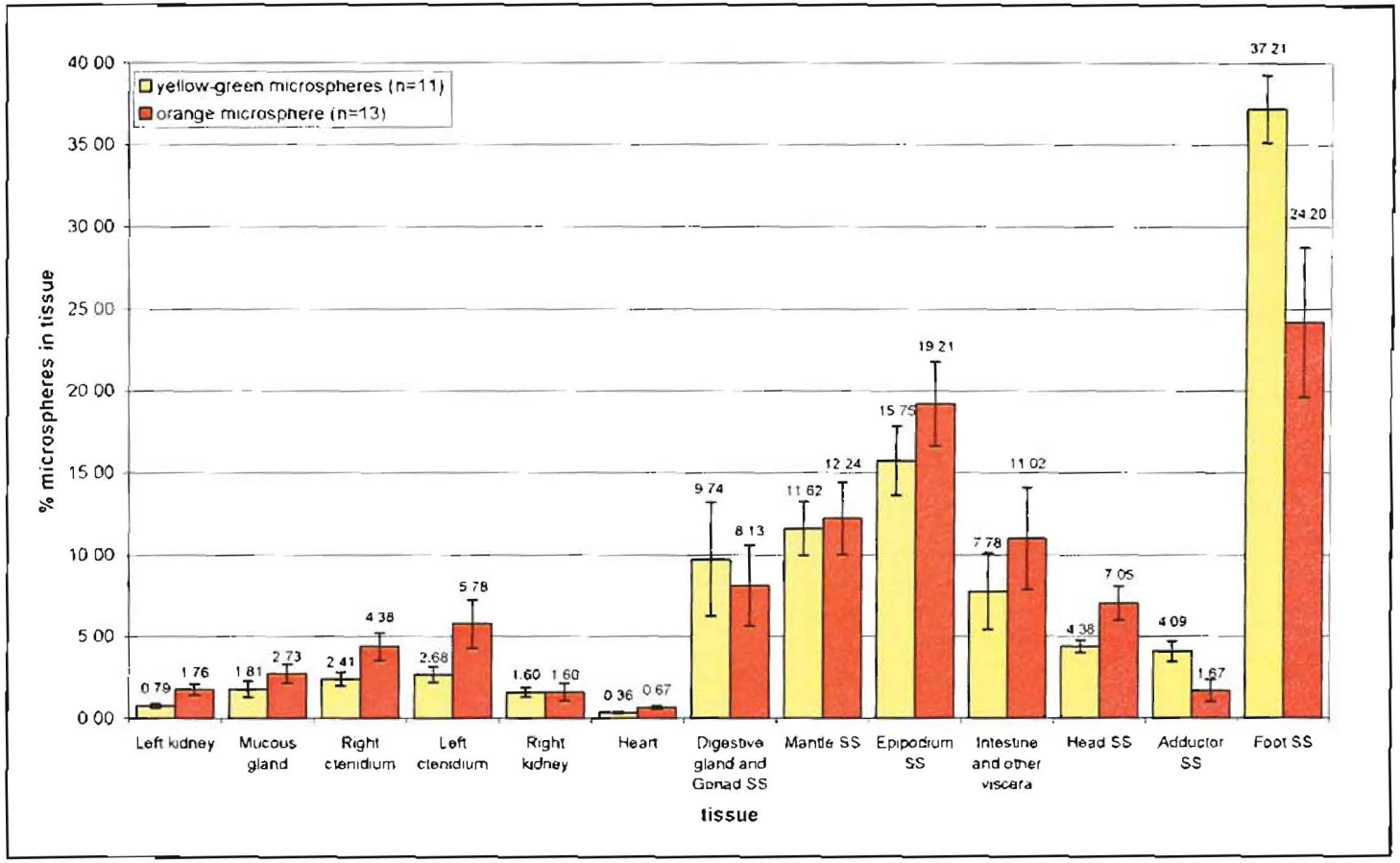


Figure 4.4. Changes in the partitioning of cardiac output among selected organs in clamping animals as indicated by distribution of two colours of fluorescent microspheres injected under resting and clamping conditions respectively. The first injection of yellow-green microspheres was into resting animals, the second injection of orange microspheres was administered under induced clamping which was maintained a further 10 minutes until they were sacrificed. The values are mean percentages of the total microsphere recovery for the respective injections and the error bars denote the standard error of the mean. The differences between the distributions of the two colours of microspheres reflect changes in the relative perfusion of tissues during the clamping state.

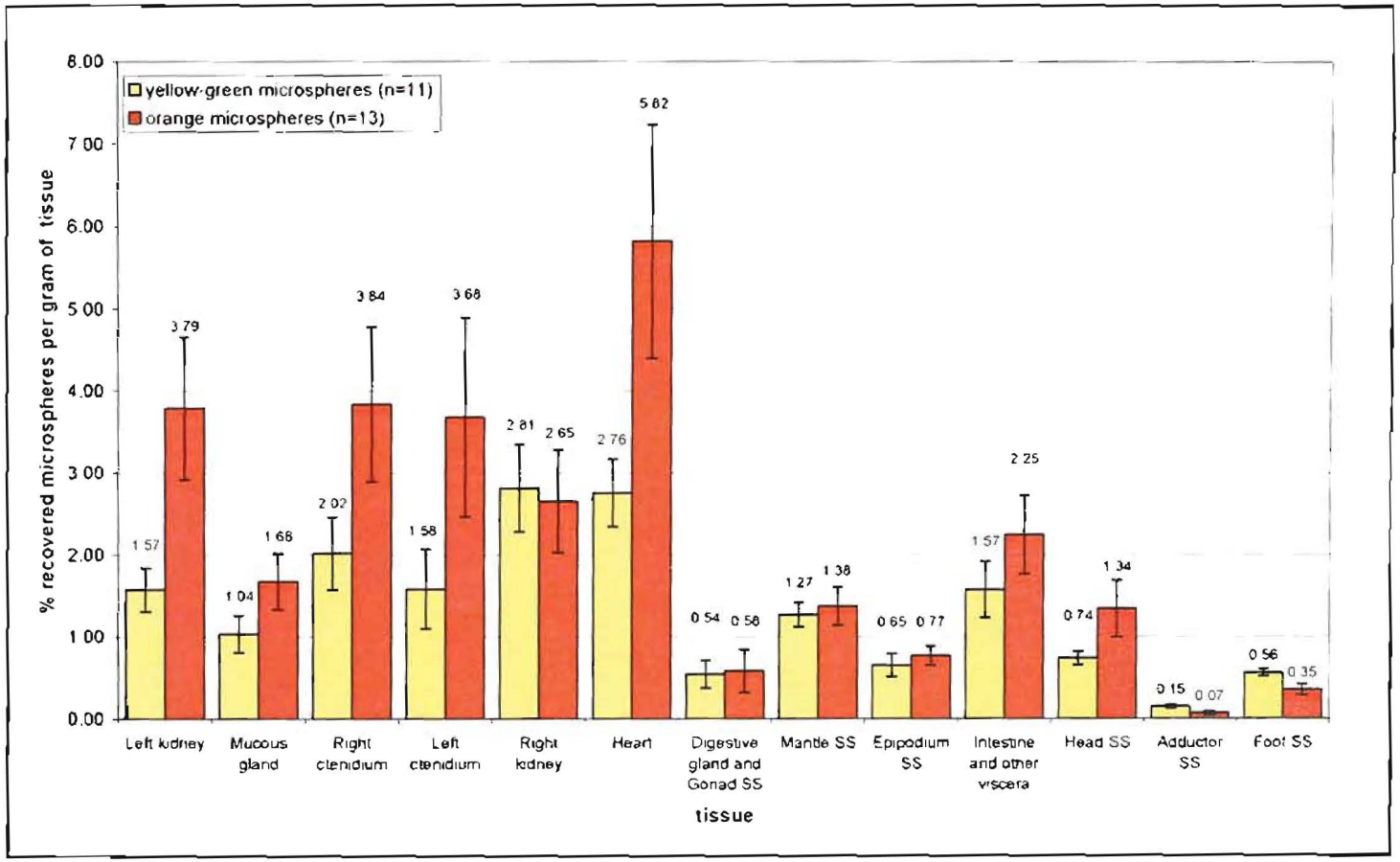


Figure 4.5. Changes in Mass specific distribution of cardiac output among selected organs in clamping animals as indicated by distribution of two colours of fluorescent microspheres injected 10 minutes apart. The first injection of yellow-green microspheres was into resting animals, the second injection of orange microspheres was administered under induced clamping which was maintained a further 10 minutes until they were sacrificed. The values are mean percentages of the total microsphere recovery per gram tissue for the respective injections and the error bars denote the standard error of the means. The differences in distribution of the two colours of microspheres reflect changes in the mass specific perfusion of tissues during clamping.

4.3.5 Changes in the partitioning of cardiac output associated with clamping in paua

The general similarity between the distributions of the microspheres from the first injection (yellow-green) in both resting and clamped animals (Figures 4.2 and 4.4) indicates that clamping itself did not cause redistribution of the microspheres. This is discussed further in chapter two.

Clamping changed the distribution of blood flow to specific tissues in *H. iris*. Increases in the relative perfusion during the clamping state were observed in the head, left and right ctenidia, left kidney, and heart, while concurrent decreases in the perfusion of the adductor and foot muscles were observed (Figures 4.4 and 4.5).
The intestine and other viscera, gonad and digestive gland, epipodium, mantle, mucous gland and interestingly the right kidney, did not exhibit changes in blood flow associated with the clamping state (Figures 4.4 and 4.5).

The majority of the blood flow in the "resting" animal (yellow-green injection) was to the foot muscle (37.2±2.08%) and this decreased noticeably during clamping (24.2±4.59%) (Figure 4.4). The foot has a comparatively low mass specific perfusion (Figure 4.5), and accordingly the flow decreased by a similar factor (Figure 4.5; YG 0.56±0.04%, OR 0.35±0.07%).

A reduction in the blood flow to the adductor muscle occurred during clamping, where less than half the relative (Figure 4.4; YG 4.1±0.62%, OR 1.7±0.68%) and mass specific perfusion (Figure 4.5; YG 0.15±0.02%, OR 0.07±0.03%) was observed.

Left kidney tissue received a comparatively high mass specific fraction of the cardiac output which increased noticeably during clamping (Figure 4.5; YG 1.57±0.27%, OR 3.79±0.87%). The left and right ctenidia both received similar yellow-green microsphere entrapment at rest (Figure 4.4; YG 2.7±0.49 left; 2.4±0.40 right), which increased, more so in the left, during clamping (Figure 4.4; OR 5.8±1.5% left; 4.4±0.87% right). The implications of this apparent arterial flow will be discussed later in this chapter.

Although there is no coronary artery in *Haliotis sp.* the heart trapped small numbers of microspheres in both resting and clamping animals (Figure 4.4). In mass specific terms the

apparent perfusion was very high in the resting animal (Figure 4.5; YG 2.76±0.41%) and this increased noticeably during the clamping treatment where it was the highest of all tissues (Figure 4.5; OR 5.82±1.42%).

The interpretation of an apparently high mass specific perfusion of the heart is discussed in chapter two. No change was observed in the right kidney between resting and clamping (Figures 4.4 and 4.5), but this tissue showed a relatively high mass specific flow for both treatments (Figure 4.5; YG 2.81±0.53%, OR 2.65±0.63%).

During the hypothesised increased arterio-venous shunting thought to occur in clamping events, one might expect to see an increase in relative perfusion of the right kidney, but this was clearly not observed and will be considered further in the discussion section of this chapter.

4.3.6 Measurement of cardiac output using a reference withdrawal technique

The heart rate averaged 21.8±1.58bpm (beats per minute) in the animals during the first injection (resting), and this decreased markedly to 15.9±2.34bpm over the duration of the clamping treatment.

Reference withdrawals were successful in three animals in both resting and clamping states and the data are summarised in tables 4.1 and 4.2. The calculations of cardiac output and stroke volume (CSV) are reported in chapter two.

Table 4.1. Measurements of cardiac parameters from reference withdrawal during injection of yellow-green microspheres into a resting animal. N.B. Heart f is the mean over the duration of the reference withdrawal sampling. Cardiac output, Q, and SV were calculated as described in chapter two.

Animal #	tissue weight (g)	reference flow rate ($ml.min^{-1}$)	Q ($ml.min^{-1}$)	mass specific Q ($ml.kg^{-1}.min^{-1}$)	heart f ($beats\ min^{-1}$)	calculated SV (ml)
M343	221	0.10	30.6	138	19.8	1.54
M346	222	0.12	23.2	105	20.2	1.15
M345	254	0.08	30.7	121	25.2	1.22

Table 4.2. Measurements of cardiac parameters from reference withdrawal during injection of orange microspheres into a clamping animal. N.B. Heart f is the mean over the duration of the reference withdrawal sampling. Cardiac output, Q, and SV were calculated as described in chapter two.

Animal #	tissue weight (g)	reference flow rate ($ml.min^{-1}$)	Q ($ml.min^{-1}$)	mass specific Q ($ml.kg^{-1}.min^{-1}$)	heart f ($beats\ min^{-1}$)	calculated SV (ml)
M343	221	0.09	13.6	62	11.7	1.17
M346	222	0.10	5.0	23	12.6	0.40
M345	254	0.13	10.5	41	21.6	0.48

The calculated cardiac output, Q, in the resting state ranged from 23.2 to 30.7ml-min^{-1} and the heart rate ranged from 19.8 (M343) to 25.2 (M345) beats per minute (Table 4.1). The CSV in the resting animals ranges from 1.15ml (M346) to 1.54ml (M343) (Table 4.1). The values of CSV are within the range of ventricular volume estimated from corrosion casts (unpublished observation).

The animals were forced to clamp during the second injection (orange microspheres) during which the cardiac output was reduced to less than one half in all animals compared with the resting state (Table 4.2; Q ranged from 5.0 to 13.6ml per minute). A concurrent decrease in CSV is observed in the clamped animals and ranges from 0.40 to 1.17ml (Table 4.2)

Table 4.3. Differences in calculated cardiac parameters after clamping.

Values are the percentage difference observed in the clamping treatment from the resting state.

Animal #	% difference in Q	% difference in heart rate	% difference in SV
M343	-55	-41	-24
M346	-78	-38	-65
M345	-66	-14	-60

Cardiac output is determined by two factors, CSV and heart rate, therefore reduction in cardiac output is a consequence of a combination of decrease in CSV, and heart rate. In animal M345 the heart rate appeared relatively unaffected by the animal clamping, decreasing only 14%, however a large apparent decrease in CSV (60%) was the main reason the cardiac output decreased 66% percent (Table 4.3)

The heart rate in animals M343 and M346 decreased similarly on clamping, about 40%, yet the CSV in M343 only decreased 24% resulting in a cardiac output decrease of 55%. Animal M346 showed a greater decrease in calculated CSV, consequently cardiac output decreased markedly (78%) (Table 4.3).

While the cardiac output shows large decreases on clamping, the CSV and heart rate decrease differentially between animals (Table 4.3). It is hard to establish whether CSV or heart rate decreases are the primary cause of the observed decrease in cardiac output. Interpretation of the heart rate monitor output during clamping is somewhat difficult, and depending on how this is done, can markedly affect the proportion of the cardiac output which is attributed to heart rate alone.

4.4 DISCUSSION

4.4.1 Blood flow during clamping

It has been suggested that the circulation of the foot and adductor muscles is nearly isolated from main circulation during sudden clamping events (Bourne and Redmond, 1977a; Russell and Evans, 1989). The microsphere data support this suggestion, with a noticeable decrease in the number of microspheres recovered from both the foot and adductor muscles when the animal was forced to clamp. The exact mechanism for the isolation of the foot and adductor tissue from the rest of the circulation is not entirely clear.

Bourne and Redmond (1977a) identified a "valve-like flap" of muscle which prevents blood flow from the CAS into the pedal and epipodial arteries in *H. corrugata* (Bourne and Redmond, 1977a), and Crofts (1929) identified a muscular valve between the venous elements of the foot and the CVS. These two valves would effectively isolate the foot from the main circulation from both the arterial and venous sides during sudden violent activity as Bourne and Redmond (1977a) mention. These valves have not been found in *H. ruber* or *H. kamtschatkana* (Russell and Evans, 1989) and the data from my microsphere experiments do not show any change in the epipodial perfusion which might be expected if a valve between the CAS and pedal and epipodial arteries, as identified by Bourne and Redmond (1977a) in *H. corrugata*, was preventing blood flow to the epipodial arteries in *H. iris*.

Another mechanism was suggested by Russell and Evans (1989) for *H. ruber*, in which extreme muscular contraction of the foot, as occurs in clamping, causes occlusion of the lacunar tissue spaces (LTS) in the foot. This generates resistance too high to permit blood flow through the tissue, resulting in ischaemia, which is known to occur in vertebrate muscle tissue as a consequence of contraction (Deveci and Eggington, 1999).

During occlusion of the pedal LTS in *H. ruber*, it was proposed that blood in the CAS would be re-directed through a shunt into the CVS and returned to the heart (Russell and Evans, 1989). The return route for from the CVS to the ctenidia is poorly understood. The right kidney receives venous blood and this constitutes a renal portal system which is perceived as the common venous drain (Crofts, 1929). From here blood is directed into the basibranchial sinus, then through the ctenidia and back into the heart (Chapter one, Figure 1.1). However, the appearance of many microspheres in the ctenidia suggests that the right kidney could be bypassed altogether to return some blood directly to the basibranchial sinus.

Morphological evidence of the vascular connection between the CAS and CVS present in *H. ruber* (Russell and Evans, 1989), and suggested by Crofts (1929) in *H. tuberculata* has thus far remained elusive in *H. iris*.

Strong support for the existence of this shunt in *H. iris* is provided by the observation of a marked increase in ctenidial microsphere entrapment during clamping. As the ctenidia lie just downstream of the right kidney with respect to circulation, we might expect to see increased microsphere entrapment in the right kidney if blood was re-directed through a CAS-CVS shunt. The results do not show increased flow to the right kidney. Alternatively, the increased microsphere recovery observed in the gills may result from an increased flow via a renal bypass as noted above.

It has been reported in the pleurotomariacean *Mikadotrochus* that some or all of the blood from the head and foot goes directly to the mantle skirt, thus bypassing the right renal organ (Andrews, 1985), but this route is not reported in *Haliotis sp.*, nor was an increased entrapment in the mantle observed.

The current abalone circulation model mentioned in chapter one (Figure 1.1) suggests that the entire systemic circulation returns via the right kidney. Thus after the passing through the

systemic circulation the right kidney receives 100 percent of the cardiac output, as do the ctenidia in series with the kidney.

It would therefore be a little misleading to express venous entrapment as a percentage of cardiac output. Instead the microsphere entrapment here provides an indication of the degree of shunting from the arterial to the venous sides of the circulation. It is worth noting that if the hypothesised CAS-CVS or renal shunts exist, they would deliver essentially oxygenated blood to the kidney or gills. The relative proportion accumulation of microspheres in the organs does in effect measure "arterial" flow to these organs, albeit arriving via a nominally "venous" route.

The increased relative blood flow in the gills during clamping is not likely to be a consequence of hypoxia, as suggested to occur by Ainslie (1980), because the duration of the clamping is relatively short. Further investigations on this are required including ventilation rates, oxygen consumption, and measurement of anaerobic metabolites during various periods of induced clamping.

4.4.2. Microsphere distribution data from *H. iris* compared with other abalone

Jorgensen et al (1984) investigated the partitioning of cardiac output in *H. cracherodii*, using 15 µm radioactively labelled microspheres. The weight distribution of the tissues reported in *H. cracherodii* is displayed in table 4.4 and is compared with the data from the present study in *H. iris* (Table 4.5).

Differences between the mass distributions of tissues samples in both studies (Tables 4.4 and 4.5) no doubt reflect differing dissection procedures as well as species differences. It appears that Jorgensen et al's study (1984) included the adductor muscle in with the foot muscle, although this is not specifically stated.

The digestive gland and gonad, a primary indication of the animals' condition, and the mantle and epipodium, comprise a greater proportion of the weight in *H. iris* than in *H. cracherodii*, whereas the amount of right kidney I was able to sample was substantially less as a percentage of the total body weight than Jorgensen et al (1984) sampled (Tables 4.4 and 4.5).

Table 4.4.

Modified from (Jorgensen et al., 1984).

Percent body weight represented by selected tissues of black abalone (n=7)

Tissue	% Body weight
gonad	6.1 (1.2)
digestive gland	5.8 (0.6)
foot muscle	66.1 (1.6)
mantle	3.3 (0.3)
radular muscle	0.8 (0.1)
right kidney	0.9 (0.1)
left kidney	0.4 (0.04)
epipodium	0.8 (0.3)

Table 4.5.

(present study)

Percent body weight represented by selected tissues of *H. iris* (n= 27)

Tissue	% Body weight
gonad + digestive gland	15.6 (1.05)
adductor	14.9 (0.82)
foot	39.1 (2.11)
mantle	5.4 (0.25)
head	3.3 (0.12)
right kidney	0.3 (0.03)
left kidney	0.3 (0.02)
epipodium	14.0 (0.30)
left ctenidium	1.1 (0.04)
right ctenidium	0.7 (0.02)
mucous gland	0.9 (0.08)
heart	0.1 (0.01)
other	4.3 (0.55)

The distribution reported by Jorgensen et al (1984) as a percentage of the cardiac output (Figure 4.6) also differs considerably from my data in resting animals described earlier (Chapter three), which had a considerably lower combined gonad and digestive gland perfusion, lower left and right kidney perfusion, and higher foot, mantle and most notably epipodial tissue perfusion.

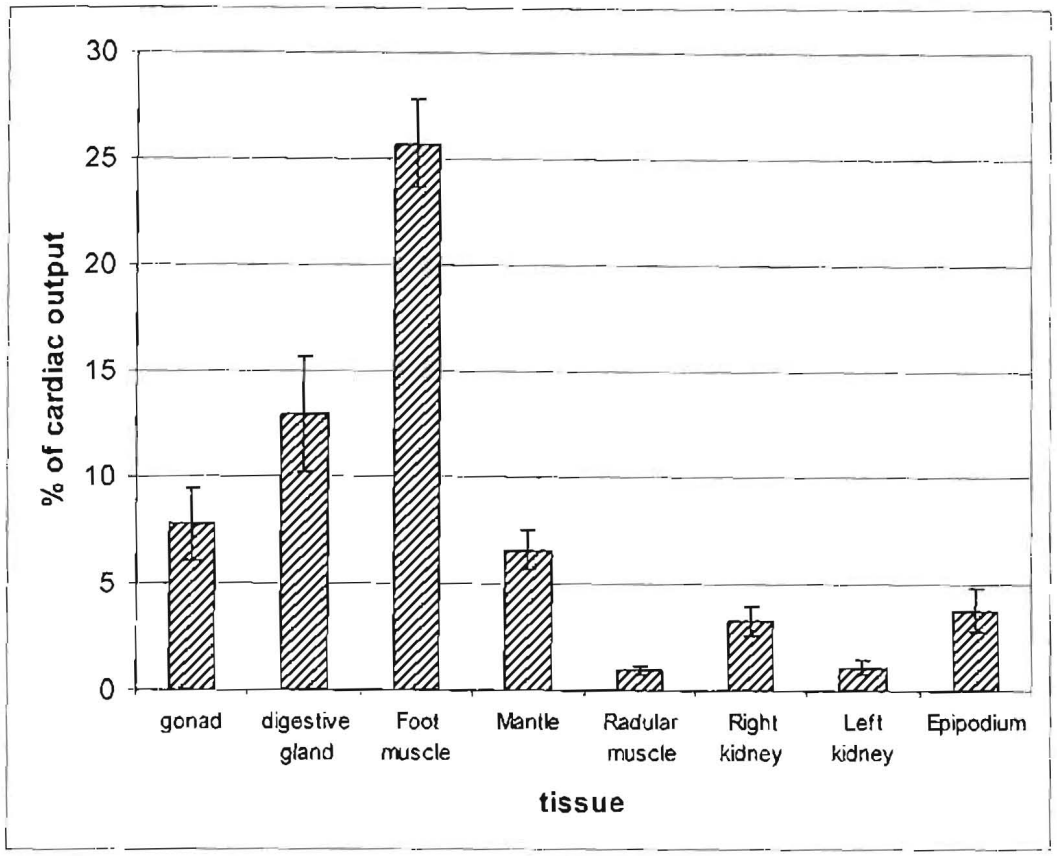


Figure 4.6. Cardiac partitioning in the black abalone, *H. cracherodii* from data by Jorgensen et al (1984).

4.4.3 Measurement of cardiac parameters using a reference withdrawal technique

The reference withdrawal method is well established as a reliable means of calculating cardiac output in a range of vertebrates and has been discussed in chapter two (Anon, 1999a; Deveci and Eggington, 1999; Jorgensen et al., 1984; Russell and Evans, 1989).

The method of passive withdrawal used was probably the most representative of a true organ as the pulsatile nature of the heart contributes to the flow (Chapter two). The heart rates of the animals at resting state in my experiments, at an average of about 22, are within the range which appears in the literature for other abalone (Bourne and Redmond, 1977a; Jorgensen et al., 1984; Krajniak and Bourne, 1988; Russell and Evans, 1989).

Jorgensen et al (1984) used a similar microsphere method of calculating cardiac output of *H. cracherodii* from which they determined the cardiac output for an animal about 200g in wet body weight at around 29 ml-min^{-1} and a mass specific flow of about 140 ml kg^{-1}·min^{-1}, and

Bourne (1974) measured a considerably lower cardiac output in two specimens of *H. corrugata*, at 39.58±0.054 and 44.12±0.728 ml $kg^{-1} \cdot min^{-1}$ (where ± is std dev of the mean), by thermal dilution.

All three reference withdrawals performed on unperturbed animals (Table 4.1) provide values similar to those obtained by Jorgensen et al (1984) in *H. cracherodii*. The latter were verified using thermal dilution measurements, but much lower values were obtained by Bourne (1974) in *H. corrugata*. The CSV calculated by Jorgensen et al (1984) in *H. cracherodii* ranges from 1ml to 1.4ml in an animal of about 200g tissue weight, is similar to that calculated in resting *H. iris* (Table 4.1), and larger than the CSV calculated by Bourne (1974) (1.026±0.054ml and 0.975±0018ml; ± is std dev of mean) in animals of considerably larger size (432 and 457g wet weight).

Table 4.6 Estimated total changes in mean tissue blood flow to sampled organs based on relative flow (Figure 4.4) and mass specific cardiac output (Tables 4.1 and 4.2) during resting and clamping states.

Tissue	flow to organ during resting (ml min^{-1})	Flow to organ during clamping (ml min^{-1})	ratio
left kidney	0.22	0.17	0.770
mucous gland	0.51	0.27	0.520
right ctenidium	0.68	0.42	0.625
left ctenidium	0.76	0.56	0.742
right kidney	0.45	0.16	0.344
heart	0.08	0.04	0.535
digestive gland and gonad	2.75	0.79	0.287
mantle	3.28	1.19	0.362
epipodium	4.44	1.86	0.420
intestine and other viscera	2.19	0.99	0.450
head	1.01	0.58	0.572
adductor	1.15	0.16	0.141
foot	10.49	2.35	0.224

The dramatic decrease in cardiac output when the animal is forced to clamp (Table 4.2) is not only due to a decrease in heart rate, but also as a result of an apparently greatly reduced CSV. Table 4.6 is derived from the data reported in this chapter and shows the considerable changes in terms of actual perfusion which occur during clamping, illustrating the advantage of using cardiac output measurements to calibrate regional perfusion measurements, thus expressing regional perfusion in terms of absolute flow. The relative distribution of the microspheres may appear similar between treatments, but table 4.6 shows that in some organs, particularly the adductor muscle and foot, there is a greatly decreased actual blood flow.

If the foot and adductor tissues are isolated from the rest of the circulation, as indeed they appear to be, then the actual cardiac output to perfuse the remaining tissues in the main circulation is greatly reduced, thus a decrease in cardiac output is necessary to prevent excessive blood flow in these tissues, a possible reason why acardia is observed during movement (Bourne et al., 1990).

CHAPTER FIVE

GENERAL DISCUSSION

The relative perfusion of the tissues of abalone during the physiological states of clamping and emersion were not previously known. The application of a technique using fluorescent microspheres in *H. iris* appeared to yield an authentic representation of relative organ perfusion in resting animals and changes in the circulatory distribution associated with emersion and clamping.

Animals which are emersed for extended periods become hypoxic (Behrens et al., 2002; Wells and Baldwin, 1995). In other gastropods it has been reported that surface tissues, other than the gills, function as auxiliary gas exchange organs during environmental hypoxia (Brown, 1984; Kingston, 1968). The observations of the present study in *H. iris* did not show any changes in blood flow to peripheral tissues as might have been expected if they were facilitating auxiliary gas exchange.

The blood flow to the foot decreased noticeably during the emersed state and taken with an increased entrapment of microspheres in the gills, supports the possibility that closure of valves in the foot isolate the foot from the rest of the circulation (Crofts, 1929) during this state. The remaining blood flow appeared to be redirected at least through a cephalo-arterial sinus (CAS) to cephalo-venous sinus (CVS) shunt, which has been found in other species of *Haliotis* (Russell and Evans, 1989). This partial redirection of blood flow seemed to supply specific tissues which are presumably less capable of anaerobic function than the foot (Baldwin et al., 1992; Wells and Baldwin, 1995).

Isolation of the foot muscle, which comprises a large proportion of the body, appeared to increase the turnover of blood to the gills and other tissues, such as the intestine and other viscera. Thus, despite an apparent decrease in heart rate observed (and thus presumably cardiac output too), flow to these tissues might have remained relatively high because fewer organs in the animal required perfusion.

The increased observed blood flow to the gills, with respect to the amount of blood being circulated in the animal during the emersed state, presumably satisfied the oxygen requirements of the remaining perfused tissues, despite an outwardly apparent decrease in functionality of the gills under aerial conditions.

In summary, the results indicated that *H. iris* copes with emersion primarily not by trying to increase gas exchange, but rather by lowering total oxygen and circulatory requirements by shutting down tissue flow to functionally hypoxic organs, most notably the foot muscle.

Future research could investigate changes in absolute blood flow (ml min^{-1}) to the tissues by measuring cardiac output during the emersed state, and also the time taken to restore normal circulatory distribution upon re-immersion in water. Respirometry, measurement of tissue specific hypoxia and oxygen diffusion, in future experiments would help us to integrate blood flow changes associated with emersion observed in the present study, with gas exchange requirements and anaerobic capacity of *H. iris*.

Clamping appeared to isolate blood flow to foot and adductor muscle from the rest of the circulation, and considerable arterio-venous shunting was suggested by the increased appearance of microspheres in the gills. These observations were consistent with closure of valves observed in other abalone (Crofts, 1929), and shunting from the CAS to the CVS (Russell and Evans, 1989) reported to occur during clamping. The relative perfusion of the adductor and foot muscles was considerably decreased by clamping, more so than was observed during emersion.

Cardiac output was measured using a reference withdrawal sampling method. Decreases of more than 50% were observed during clamping compared to resting (Table 4.3). The reduction in cardiac output was due to decreases both in heart rate and cardiac stroke volume (Table 4.3). Thus, the observed decrease in relative perfusion of the adductor and foot muscle tissues during clamping actually correspond to a larger decrease in tissue blood flow (ml min^{-1}), estimated at about 20% of that in the resting animal (Table 4.6; adductor muscle). Correspondingly the blood flow to the gills did not decrease as markedly, and during clamping was estimated at not less than 50% of that in the resting animal.

Further studies are required to identify the exact mechanisms which isolate the foot and adductor muscles from the rest of the circulation during clamping, and to find the apparent vascular connection between the CAS and CVS which has thus far remained elusive in *H. iris*.

It is not known how long animals can maintain forceful clamping to the substrate, but because of its primary function as a defense mechanism, and the considerable changes which it causes in the circulatory system, it is probable that its use is designed to be relatively brief in duration.

Further understanding of handling stress and circulatory changes associated with emersion and clamping would be of benefit to the commercial abalone industry, where handling, live transport under emersed conditions and recovery from these physiological states is paramount.

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REFERENCES

Ainslie, A. 1980 The quantitative role of haemocyanin in the respiration of abalone (genus *Haliotis*) *Journal of Experimental Zoology*. 211:87-99.

Allen, V.J., I.D. Marsden, and N.L.C. Ragg. 2001 The use of stimulants as an aid to wean fishery caught blackfoot abalone (*Haliotis iris*) to artificial food. *Journal of Shellfish Research*. 20:647-651.

Andrew, N.L., P.A. Breen, J.R. Naylor, T.H. Kendrick, and P.K. Gerring. 2000. Stock assessment of paua (*Haliotis iris*) in PAU 7 in 1998-99. New Zealand Fisheries Assessment Report. Ministry of Fisheries. 40.

Andrews, E.B. 1985. Structure and function in the excretory system of archaegastropods and their significance in the evolution of gastropods. *Philosophical Transansactions of the Royal Society of London*. 310:383-406.

Anon. 1999a Manual for using fluorescent microspheres to measure regional organ perfusion. Fluorescent Microsphere Resource Center. University of Washington. Division of Pulmonary and Critical Care Medicine, Washington.

Anon. 1999b. Fluospheres® fluorescent microspheres for blood flow determination. Technical bulletin MP08829. Molecular Probes. 6.

Anon. 1999c. Sedimentation method for determination of organ blood flow with fluorescent microspheres. Technical bulletin X08829. Molecular Probes 2

Baldwin, J., R.M.G. Wells, M. Low, and J.M. Ryder. 1992 Tauropine and D-lactate as metabolic stress indicators during transport and storage of live paua (New Zealand abalone) (*Haliotis iris*) *Journal of Food Science*. 57:280-282.

Barron, M.G., B.D. Tarr, and W.L. Hayton. 1987. Temperature-dependence of cardiac output and regional blood flow in rainbow trout, *Salmo gairdneri* Richardson. *Journal of Fish Biology*. 31:735-744.

Behrens, J.W., J.P. Elias, H.H. Taylor, and R.E. Weber. 2002. The archaeogastropod mollusc *Haliotis iris*. tissue and blood metabolites and allosteric regulation of haemocyanin function. *Journal of Experimental Biology*. 205:253-263.

Bourne, G.B. 1974. Hemodynamics in the Pink Abalone, *Haliotis corrugata* (Mollusca, Gastropoda). *Ph.D. Thesis*. Iowa State University, Ames, Iowa. 111.

Bourne, G.B., and J.R. Redmond. 1977a. Hemodynamics in the pink abalone, *Haliotis corrugata* (Mollusca, Gastropoda). Part 1-Pressure relations and pressure gradients in intact animals. *Journal of Experimental Zoology*. 200:9-16.

Bourne, G.B., and J.R. Redmond. 1977b. Hemodynamics in the Pink Abalone, *Haliotis corrugata* (Mollusca, Gastropoda). Part 2-Acute blood-flow measurements and their relationship to blood pressure. *Journal of Experimental Zoology*. 200:17-22.

Bourne, G.B., J.R. Redmond, and D.D. Jorgensen. 1990. Dynamics of the Molluscan circulatory system: Open versus closed. *Physiological Zoology*. 63:140-166.

Brown, A.C. 1984. Oxygen diffusion into the foot of the whelk *Bullia digitalis* (Dillwyn) and its possible significance in respiration. *Journal of Experimental Marine Biology and Ecology*. 79:1-7

Cameron, J.N. 1974. Blood flow distribution as indicated by tracer microspheres in resting and hypoxic arctic grayling (*Thymallus arcticus*). *Comparative Biochemistry and Physiology*. 52A:441-444.

Crofts, D.R. 1929. *Haliotis*. LMBC Memoirs on Typical British Marine Plants and Animals No. 29. Hodder and Stoughton Ltd, London. 174 pp

Deveci, D., and S. Eggington. 1999. Development of the fluorescent microsphere technique for quantifying regional blood flow in small mammals. *Experimental Physiology*. 84:615-630

Glenny, R., S. Bernard, and W. Lamm. 2000. Hemodynamic effects of 15μm diameter microspheres on the rat pulmonary circulation. *Journal of Applied Physiology*. 89:499-504

Glenny, R., S. McKinney, and K. Robertson. 1997. Spatial pattern of pulmonary blood flow distribution is stable over days. *Journal of Applied Physiology*. 82:902-907.

Gordon, H.R., and P.A. Cook. 2001. World abalone supply, markets and pricing: historical, current and future. *Journal of Shellfish Research*. 20:567-570

Jackson, D., K.C. Williams, and B.M. Degnan. 2001. Suitability of Australian formulated diets for the aquaculture of the tropical abalone *Haliotis asinina* Linnaeus. *Journal of Shellfish Research*. 20:627-636

Jorgensen, D.D., S.K. Ware, and J.R. Redmond. 1984. Cardiac output and tissue blood flow in the abalone, *Haliotis cracherodii* (Mollusca, Gastropoda). *Journal of Experimental Zoology*. 231:309-324.

Kendrick, T.H., and N.L. Andrew. 2000. Catch and effort statistics and a summary of standardised CPUE indices for paua (*Haliotis iris*) in PAU 5A, 5B, and 5D. New Zealand Fisheries Assessment Report. Ministry of Fisheries. 25.

Kingston, R.S. 1968. Anatomical and Oxygen Electrode Studies of Respiratory Surfaces and Respiration in *Acmea*. *The Veliger*. 11 Supplement:73-78.

Krajniak, K.G., and G.B. Bourne. 1987. Effects of FMRFamide on the Intact and Isolated Circulatory System of the Pinto Abalone, *Haliotis kamtschatkana*. *Journal of Experimental Zoology*. 241.

Krajniak, K.G., and G.B. Bourne. 1988. Aortic and venous blood pressures in the intact pinto abalone, *Haliotis kamtschatkana*. *Comparative Biochemistry and Physiology* 89A:405-407.

Krajniak, K.G., and G.B. Bourne. 1989. The effects of 5-Hydroxytryptamine on the isolated and intact circulatory system of the pinto abalone, *Haliotis kamtschatkana*, and its presence in cardiac and non-cardiac tissues. *Comparative Biochemistry and Physiology*. 94C:561-566.

Ministry of Fisheries. 2002. Summary of the assessments of the sustainability of current TACCs and recent catch levels and status of the stocks for the 2000-2001 fishing year. Vol. 2002. Ministry of Fisheries.

Mulder, A.L.M., C.A.V. Goor, D.A. Giussani, and C.E. Blanco. 2001. α-Adrenergic contribution to the cardiovascular response to acute hypoxemia in the chick embryo. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. 281:R2004-R2010.

Poore, G.C.B. 1972a. Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 1. Feeding. *New Zealand Journal of Marine and Freshwater Research*. 6:11-22.

Poore, G.C.B. 1972b. Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 2. Seasonal and diurnal movement. *New Zealand Journal of Marine and Freshwater Research*. 6:246-258.

Poore, G.C.B. 1972c. Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 3. Growth. *New Zealand Journal of Marine and Freshwater Research*. 6:534-559.

Poore, G.C.B. 1973. Ecology of New Zealand abalones, *Haliotis* species (Mollusca: Gastropoda) 4. Reproduction. *New Zealand Journal of Marine and Freshwater Research*. 7:67-84.

Prinzen, F.W., and J B. Bassingthwaighte. 2000. Blood flow distributions by microsphere deposition methods. *Cardiovascular Research*. 45:13-21.

Russell, C W , and B.K. Evans. 1989. Cardiovascular Anatomy and Physiology of the Black-Lip Abalone, *Haliotis ruber*. *Journal of Experimental Zoology*. 252:105-117.

Sainsbury, K.J. 1982a. Population dynamics and fishery management of the paua, *Haliotis iris* I Population structure, growth, reproduction, and mortality. *New Zealand Journal of Marine and Freshwater Research*. 16:147-161.

Sainsbury, K.J. 1982b Population dynamics and fishery management of the paua, *Haliotis iris* II. Dynamics and managements as examined using a size class population model. *New Zealand Journal of Marine and Freshwater Research*. 16:163-173.

Schiel, D.R 1992. The paua (abalone) fishery of New Zealand. *In* Abalone of the World. Biology, Fisheries, and Culture. S.A Sheperd, M.J. Tegner, and S.G.d. Proo, editors. Blackwell Scientific, Oxford 276-304

Schiel, D.R , and P.A. Breen. 1991. Population Structure, Ageing, and Fishing Mortality of the New Zealand Abalone *Haliotis Iris*. *Fishery Bulletin (U.S.)*. 89:681-691

Schimmel, C , D Frazer, and R. Glenny. 2001 Extending fluorescent microsphere methods for regional organ blood flow to 13 simultaneous colors. *American Journal of Physiology - Heart and Circulatory Physiology*. 280:H2496-H2506.

Servier-Zaragoza, E., A Mazariegos-Villareal, G. Ponce-Díaz, and S Montes Magallón. 2001. Growth of juvenile abalone, *Haliotis fulgens* Philippi, fed different diets. *Journal of Shellfish Research* 20:689-693

Shipton, T A., and P.J. Britz. 2001. The partial and total replacement of fishmeal with selected plant protein sources in diets for the South African abalone, *Haliotis midae* L. *Journal of Shellfish Research*. 20:637-645.

Shultz, I.R , M G. Barron, M C. Newman, and A.M Vick. 1999 Blood flow distribution and tissue allometry in channel catfish. *Journal of Fish Biology*. 54:1275-1286.

Taylor, H H., and P. Greenaway. 1984 The role of the gills and branchiostegites in gas exchange in a bimodally breathing crab, *Holthusana transversa*: Evidence for a facultative change in the distribution of the respiratory circulation. *Journal of Experimental Biology*. 111:103-121.

Taylor, J , H.H. Taylor, and D.R. Shiel. 1994. The first cut is the deepest Wounding, bleeding and healing in the black-footed paua (*Haliotis iris*). *Seafood New Zealand*. 2:47-48.

Taylor, J.E 1993. Responses to Wounding Resulting in Haemorrhage in the Black Footed Paua: *Haliotis iris* Gmelin (Mollusca, Gastropoda). *M.Sc. Thesis*. University of Canterbury, Christchurch 110.

Van Oosterhout, M F M., H.M.M. Willigers, R.S Reneman, and F W. Prinzen. 1995. Fluorescent microspheres to measure organ perfusion: validation of a simplified sample processing technique. *American Journal of Physiology - Heart and Circulatory Physiology*. 38:H725-H733

Wells, R.M G., and J. Baldwin. 1995. A comparison of metabolic stress during air exposure in two species of New Zealand abalone, *Haliotis iris* and *Haliotis australis*: implications for the handling and shipping of live animals *Aquaculture* 134:361-370.

Withers, P C. 1992. Comparative Animal Physiology Saunders College Publishing.